

**Airway remodelling in smokers with or without chronic
obstructive pulmonary disease (COPD) and the effects
of inhaled corticosteroids on remodelling in COPD**

By

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Submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy (PhD)

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Declaration of Originality

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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Abstract

Introduction: Smoking-related COPD is a worldwide health problem. Airway remodelling is defined as structural changes occurring in chronic inflammatory diseases of the airways. Our knowledge about airway remodelling in COPD is very limited. My preliminary observational study of bronchial biopsies (BB) from COPD subjects revealed reticular basement membrane (Rbm) fragmentation and vascular changes. I hypothesised that these changes are specific for COPD and are related to the angiogenic activity of vascular endothelial growth factor (VEGF) and transforming growth factor- β (TGF- β). I also aimed to study the effects of inhaled corticosteroids (ICS) on these airway changes.

Methods: A cross-sectional study compared BB from current smokers with COPD (S-COPD), ex-smokers with COPD (ES-COPD), current smokers with normal lung function (S-N) and healthy nonsmoking (H-N) subjects. BB were stained with anti-Collagen IV, anti-VEGF and anti-TGF- β antibodies. Rbm fragmentation and vessels in the Rbm and lamina propria (LP) were measured. Anti-Factor VIII antibody was compared with anti-Collagen IV antibody in detecting vessels.

Then a double-blind, randomized and placebo controlled study assessed the effects of ICS on airway remodeling, VEGF and TGF- β in COPD.

Results: Airway remodelling changes were also detectable in S-N. The Rbm was fragmented. The length of splits was significantly greater in both COPD groups and in S-N than controls ($p<0.02$). The Rbm was hypervascular and the LP hypovascular in current smokers compared with controls ($p<0.05$). Vessels stained for VEGF and TGF- β were increased in the Rbm of both COPD groups and S-N ($p<0.05$). Factor VIII

antibody confirmed my finding of hypovascularity of the LP in S-COPD. ICS reversed Rbm splitting but did not have any effect on vessel remodelling and angiogenic activities.

Discussion: My studies revealed novel aspects of Rbm and vascular remodelling in BB from COPD subjects and S-N and for the first time showed that ICS are effective on Rbm changes in COPD. Rbm fragmentation, we think, is probably a consequence of the effects of proteolytic enzymes on the Rbm due to activation of epithelial-mesenchymal transition (EMT) by smoking. This is under more investigation in our group. My study could not explain the mechanisms to vessel changes in current smokers. Further studies to examine the role of other angiogenic/antiangiogenic factors are now needed. Absence of vascular changes in ES-COPD subjects may imply that vascular remodelling is reversible with smoking cessation, but to test this we need a longitudinal smoking cessation study.

Publications

Publications:

- **Amir Soltani**, David W Reid, Sukhwinder S Sohal, Richard Wood-Baker, Steve Weston, H. Konrad Muller and E. Haydn Walters “*Basement membrane and vascular remodelling in smokers and chronic obstructive pulmonary disease: a cross-sectional study.*” **Respiratory Research**, 2010 July 30, 11 (1):105. doi: 10.1186/1465-9921-11-105
- Sukhwinder S. Sohal, David Reid, **Amir Soltani**, Chris Ward, Steven Weston, H. Konrad Muller, Richard Wood-Baker, E. Haydn Walters “*Reticular basement membrane fragmentation and potential epithelial mesenchymal transition is exaggerated in the airways of smokers with chronic obstructive pulmonary disease.*” Accepted for publication in **Respirology**, August 2010, 15 (6), in press. [Original article]
- Walters EH, Reid D, **Soltani A**, Ward C. “*Angiogenesis: A potentially critical part of remodelling in chronic airway disease?*” **Pharmacology and Therapeutics**, 2008, April; 118 (1): 128-37. Epub February 15 2008; doi: 10.1016/j.pharmthera.2008.01.007. [Review article]
<http://www.sciencedirect.com/science/journal/01637258>
- Walters EH, **Soltani A**, Reid DW, Ward C. “*Vascular remodeling in asthma.*” **Current Opinion in Allergy and Clinical Immunology**, 2008; 8:39-43. [Review article]
<http://journals.lww.com/co-allergy/pages/default.aspx>

Conference Presentations and Abstracts

Abstracts:

- **Amir Soltani**, Sukhwinder S. Sohal, David Reid, Steve Weston, H. Konrad Muller, Richard Wood-Baker, E. Haydn Walters. “*Airway angiogenesis and basement membrane remodelling in smokers and chronic obstructive pulmonary disease: cross-sectional and longitudinal studies.*” **American Journal of Respiratory and Critical Care Medicine** 2010; 181:A3874. This abstract was presented as a poster on Monday 17 in the American Thoracic Society (ATS) International Conference 14-19 May 2010 in New Orleans, U.S.A. <https://cms.psav.com/cAbstract/itinerary/>
- **Amir Soltani**, Sukhwinder S Sohal, David Reid, Steve Weston, HK Muller, Richard Wood-Baker, EH Walters. “*Basement membrane remodelling in COPD responds to inhaled corticosteroids.*” **Respirology** 2010, 15: supplement 1, A33. (doi: 10.1111/j.1400-1843.2010.01735.x.) This abstract was a spoken presentation (TO 090) in the TSANZ Annual Meeting 20-24 March 2010 in Brisbane, Australia.
<http://www3.interscience.wiley.com/cgi-bin/fulltext/123305339/PDFSTART>
- SS Sohal, DW Reid, **A Soltani**, C Ward, S Weston, HK Muller, R Wood-Baker, EH Walters. “*Smoking has potential to initiate epithelial mesenchymal transition (EMT) in the airway mucosa.*” **Respirology** 2010, 15: supplement 1, A32. (doi: 10.1111/j.1400-1843.2010.01735.x) This abstract was a spoken presentation (TO 086) by Mr. S.S. Sohal in the TSANZ Annual Meeting 20-24 March 2010 in Brisbane, Australia.
<http://www3.interscience.wiley.com/cgi-bin/fulltext/123305339/PDFSTART>
- **A Soltani**, S Sohal, D Reid, HK Muller, S Weston, R Wood-Baker, EH Walters. “*Inhaled corticosteroids (ICS) in airway remodelling in COPD.*” **Thorax**, 2009; 64: Supplement IV, A55, S107. This abstract was a spoken presentation by Professor E. Haydn Walters in the British Thoracic Society (BTS) Winter Meeting 2-4 December 2009, London, England.
http://thorax.bmj.com/content/64/Suppl_4/A54.full.pdf
- **Soltani**, D. Reid, S.S. Sohal, H.K. Muller, S. Weston, R. Wood-Baker, E.H Walters. “*Vascular and basement membrane remodeling in smokers and COPD.*” **European Respiratory Journal** 2009; 34: supplement 53, 48s. [Abstract]. This abstract was presented as an E-communication in the

19th European Respiratory Society (ERS) annual congress, Vienna, Austria, September 2009, and is available as E-communication in the European Respiratory Society website at http://www.ersnet.org/learning_resources_player/abstract_print_09/main_frameset.htm, Session 58, E408, page 48s.

- S.S. Sohal, D. Reid, **A. Soltani**, C. Ward, S. Weston, H.K. Muller, R. Wood-Baker, E.H. Walters. “*Smoking has potential to initiate basement membrane disruption and epithelial mesenchymal transition in COPD.*” **European Respiratory Journal** 2009; 34: supplement 53, 264s. [Abstract]. This abstract was an oral presentation by Mr. Sukhwinder (Romy) S. Sohal in the 19th European Respiratory Society (ERS) annual congress, Vienna, Austria, September 2009 and is available in the European Respiratory Society website at http://www.ersnet.org/learning_resources_player/abstract_print_09/main_frameset.htm, Session 157, 1603, page 264s, September 2009, Vienna, Austria.

Abstract listed below is not directly related to the content of my thesis:

- **Soltani A**, Reid D, Almond I, Walters EH, Wood-Baker R. “*Survey of co-morbidities in acute exacerbations of chronic obstructive pulmonary disease.*” **Respirology** 2009; 14: Supplement 1, A53. [Abstract]. This abstract was presented as a poster in the TSANZ Annual Scientific Meeting in Darwin, April 2009. <http://www3.interscience.wiley.com/cgi-bin/fulltext/122257085/PDFSTART>

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Amir Soltani

Abbreviations and symbols

Abbreviations

AECOPD = Acute exacerbations of COPD

AHR = Airway hyper-responsiveness, same as BHR

AM = Alveolar macrophage

ATS = American Thoracic Society

BAL= Broncho-alveolar lavage

BALF = Broncho-alveolar lavage fluid

BB = Per-bronchoscopic bronchial biopsies

BDR = Bronchodilator responsiveness

BM = Basement membrane

bFGF = Basic fibroblast growth factor

BHR = Bronchial hyper-responsiveness

CD (#) (like CD31 etc.) = Are surface antigens that are detectable by using different antibodies and are used to address different kinds of cells in the hematopoietic and tissue mononuclear-macrophage cellular system.

Cm = centimeter

CoR = Coefficient of repeatability

CT-scan = Computerized tomography scan

Dmin = The dose of methacholine that provokes 20% decrease in FEV1. It shows the presence and the severity of BHR.

ECM = Extracellular matrix

ELISA = Enzyme linked immunosorbent assay

EMT = Epithelial-mesenchymal transition

ERS = European Respiratory Society

ES-COPD = Exsmoker COPD

FEV1 = Forced expiratory volume in first second

FER = Forced expiratory ratio = $FEV1/FVC \text{ ratio} \times 100$

FOB = Fiberoptic bronchoscope

FP = Fluticasone propionate

FVC = Forced vital capacity

DNA = deoxy ribonucleic acid

GOLD = Global Initiative for Chronic Obstructive Lung Disease

GR = Glucocorticoid receptor

GM-CSF = Granulocyte-macrophage colony stimulating factor

H-N = Healthy and nonsmoker

HRCT = High resolution computerized scan

Hyper- = A prefix that means increase of, e.g. hypervascularity means increased vessels

Hypo- = A prefix that means decrease of, e.g. hypovascularity means decreased vessels

IC = Inspiratory capacity

ICS = Inhaled corticosteroid/inhaled corticosteroids

I- κ B = Inhibitor of κ B

IL = Interleukin

LABA = Long-acting beta-adrenergic agonist

LoA = Limits of agreement

LP = lamina propria

MAPK = Mitogen-activated protein kinase

mg = milligram

mm = millimeter

MMP = Matrix metalloproteinase

mRNA = Messenger ribonucleic acid

NF- κ B = Nuclear factor-kappa B

NO = Nitric oxide

PCR = Polymerase chain reaction

PEF = peak expiratory flow

Percent vascularity = Area of vessels/area of the lamina propria examined

Pi = Protease inhibitor = Alpha1-antitrypsine

PI3K = Phosphoinositide 3 kinases

PMN = Polymorphonuclear leukocyte

Rbm = Reticular basement membrane

RNA = Ribonucleic acid

ROS = Reactive oxygen species

S-COPD = Current smoker COPD

SD = Standard deviation

SE = Standard Error

SFC = Salmeterol + fluticasone propionate

SGRQ = St George's Respiratory Questionnaire

S-N = Smokers with normal lung function

SNP = Single nucleotide polymorphism

TGF- β = Transforming growth factor-beta

TIMP = Tissue inhibitor of metalloproteinases

Vascular density = Number of vessels/ area of the lamina propria examined

%vascular area = Percent vascular area

VEGF = Vascular endothelial growth factor

VEGFR = Vascular endothelial growth factor receptor

VEGFR-1 = Flt-1, fms-like tyrosine kinase

VEGFR-2 = KDR/FLK-1, Kinase-insert domain receptor/fetal liver kinase

VEGFR-3 = Flt4, fms-like tyrosine kinase 4

Symbols:

α = Alpha

β = Beta

γ = Gamma

μ = Micro

μm = Micrometer

ν = Nu

κ = Kappa

I = one

II = Two

III = Three

IV = Four

V = Five

VIII = Eight

FOR:

Parisa, Ehsan and Sara:

- My love, family and life.

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Appendix: Examiners' Comments

Chapter One

Introduction, Preliminary Data and Resulting Aims and Hypotheses

Introduction:

Chronic obstructive pulmonary disease (COPD) and asthma are common chronic airway diseases world-wide and in Australia (K. R. Chapman et al., 2006) (ABS, 1998; Halbert, Isonaka, George, & Iqbal, 2003; Masoli, Fabian, Holt, & Beasley). During the last two decades, asthma has been under vigorous investigation and new findings have changed our understanding of the pathogenesis of the disease and our approach to its management (Eugene Haydn Walters, Reid, Soltani, & Ward, 2008). On the other hand, COPD has been relatively neglected during this time and mostly regarded as a smoking-related self-inflicted nonreversible disease (K. R. Chapman et al., 2006; Eugene Haydn Walters et al., 2008). Our understanding of its pathogenesis and pathology has not changed much since the early second half of the twentieth century, when the protease-antiprotease explanation of the parenchymal component of the disease (emphysema) was born. Since then, there has been especially very little research on airway involvement. However, COPD continues to be a worldwide health problem that is going to be a major

challenge for the coming decades even if total smoking abatement is achieved worldwide (K. R. Chapman et al., 2006; GOLD, 2007), which is extremely unlikely.

In this chapter I will explain why our research group has been interested in airway remodelling in COPD and briefly present previous published data on this subject. The Respiratory Research Group at Menzies Research Institute and University of Tasmania has been involved in research on airway remodelling for some years and has published several articles about this in asthma, as well as inflammatory changes in the airway wall in both asthma and COPD. With this background of experience, I decided to investigate aspects of airway remodelling in large airway endobronchial biopsies in COPD. I designed and carried out a preliminary pilot study to compare current smokers with COPD with controls mainly, to get some perspective on the condition. This pilot study revealed remarkably novel findings of abnormalities in the reticular basement membrane (Rbm) and vascular changes. Based on these preliminary findings, I decided to investigate whether the changes we found were specific for COPD or if they were more generally smoking-related irrespective of the subject's lung function status. In addition, I designed a longitudinal study to evaluate the effects of anti-inflammatory treatment on airway remodelling in COPD.

Background:

Smoking is a world-wide health problem and is the main cause of COPD, as well as a major contributor to coronary and peripheral vascular disease, stroke and cancer. Nevertheless, there are only a few recently published studies in the pathogenesis and pathologic characteristics of smoking related airway disease, especially in the proximal airways (K. R. Chapman et al., 2006) (Halbert et al., 2003) (Bergeron & Boulet, 2006; James & Wenzel, 2007; Eugene Haydn Walters et al., 2008). Most of the seminal publications describing the pathology in the airways in COPD are from many decades ago. These reported squamous metaplasia and goblet cell hyperplasia of the epithelium and mucous gland hyperplasia (A. Nagai, West, Paul, & Thurlbeck, 1985; A. Nagai, West, & Thurlbeck, 1985; L. Reid, 1960). Apart from some data on the characteristics of inflammatory changes in airway mucosa, little has been added to this classic

pathological knowledge since then. Moreover, those studies that have looked for airway remodelling changes in COPD, have for the major part used surgical specimens to study parenchymal and small airway changes in patients with co-existing local lung cancer, and largely ignored large airways (Hashimoto, Tanaka, & Abe, 2005; Kasahara et al., 2001; Kranenburg, de Boer, Alagappan, Sterk, & Sharma, 2005; Kuwano et al., 1993). Then, there are few studies that have used per-bronchoscopic bronchial biopsies (BB) to study vascularity of large airways in COPD. But the results from these studies are of limited value because they only focused on vessel changes and angiogenic factors in the lamina propria (LP), while I have now found that there is substantial change in relation to the Rbm, and because they are only applicable to some specific subgroups of COPD that were included. One study in Italy for example, recruited only subjects who were smokers and had clinical criteria of chronic bronchitis with or without airway obstruction and compared them with nonsmoking controls (Calabrese et al., 2006). None of the smokers had emphysema in this study. Another group (Zanini et al., 2009) studied subjects with moderate to severe COPD, but they had quit for more than 10 years.

In contrast to COPD, there are many reports published about asthma and airway remodelling (structural changes that happen in the airway wall in the course of chronic airway diseases) in recent decades (see chapter two) (Boulet & Sterk, 2007) (Elias, Zhu, Chupp, & Homer, 1999). The most prominent findings in the mucosa of airways of asthmatics have been epithelial fragility and shedding (Dunnill, 1960; Naylor, 1962) (Rennard, 1996), Rbm thickening (Boulet et al., 1997) (Jeffery et al., 1992) (Roche, Beasley, Williams, & Holgate, 1989), eosinophilic inflammation of mucosa (Dunnill, 1960), hypervascularity of the LP (Dunnill, 1960; Li & Wilson, 1997; Salvato, 2001) (Orsida et al., 1999) and muscle hypertrophy (Carroll, Elliot, Morton, & James, 1993) (Dunnill, Massarella, & Anderson, 1969) (Bousquet, Jeffery, Busse, Johnson, & Vignola, 2000).

COPD is a pan-airway disease but there is little information about remodelling in the large airways compartment. It has been shown that large airway biopsies via a fiber-optic bronchoscope (FOB) in diseases with pan-airway involvement, such as post lung

transplant bronchiolitis obliterans syndrome (BOS), can be very informative (C. Ward et al., 1997; Chris Ward et al., 1998). With this background, I decided to examine airway biopsies taken with a FOB from COPD subjects and evaluate them for airway remodelling.

Preliminary study: Characteristics of airway remodelling in COPD

My preliminary study was a cross-sectional study designed to get some perspective on potential hallmarks of airway remodelling in BB from the airways of COPD subjects. During a preliminary scanning of the slides from current smokers with COPD and normal controls, I observed that the morphology of the Rbm and the distribution of vessels in the mucosa of COPD subjects were abnormal.

As a result of those very first observations, I *hypothesised* that Rbm morphology and vascular distribution are different in COPD compared to normal. To test this hypothesis I focused in my first formal but preliminary study on these two components of airway remodelling.

Hypotheses in the pilot study:

1. The morphology of the Rbm is different in COPD compared with control subjects.
2. The distribution of vessels in the LP and in relation to the Rbm is different in COPD compared with control subjects.

Methods:

See chapter three for details of methods. An outline is only given here.

Study design and Subjects: This preliminary cross-sectional study compared BB from current smokers with physiological evidence of COPD (S-COPD) with healthy nonsmokers (H-N). This was done in a blinded and randomised manner. Individual slides were coded by an independent person and their assessment and quantification(s) were done with no prior knowledge of the origin or diagnosis.

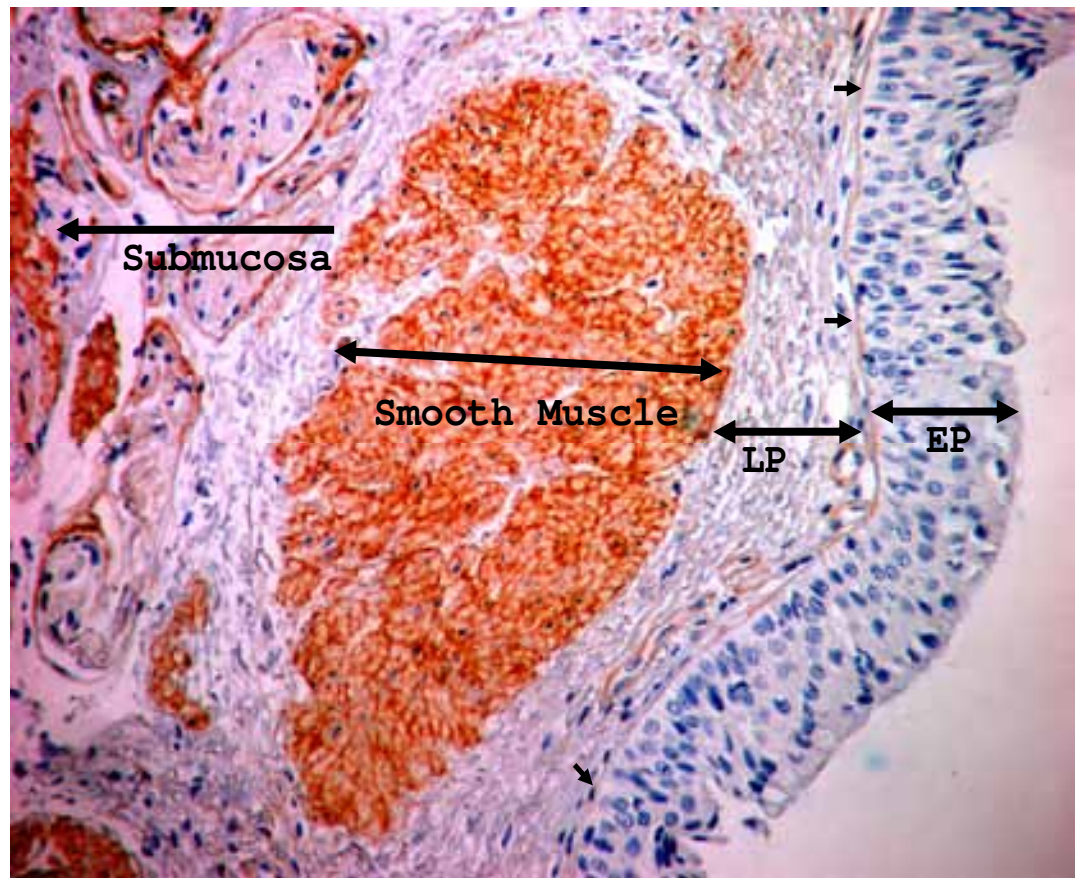


Figure 1-1. *Microscopic anatomy of airway biopsy taken by FOB.* The epithelium (EP) covers the most superficial part of mucosa and is exposed to airway lumen. It is based and attached on the basement membrane (marked by arrows) which is a linear structure. The lamina propria (LP) is made up of loose connective tissue and contains blood vessels. The deepest part of the mucosa is smooth muscle. Some submucosa may sometimes be seen in BB and is mainly composed of mucous glands and loose connective tissue. BB from normal subject. Collagen IV antibody, x200.

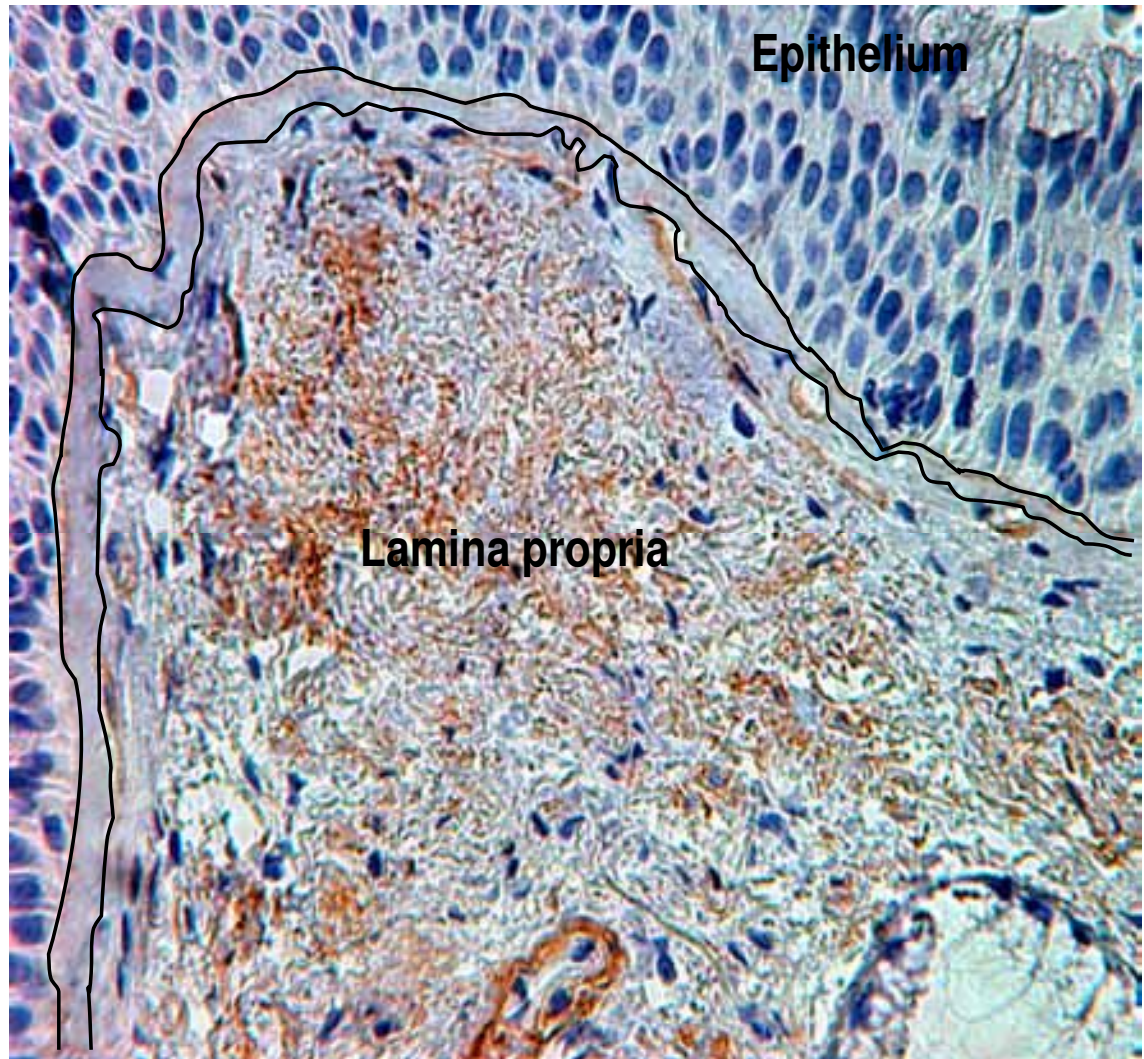


Figure 1-2. *Measuring Rbm thickness.* The Rbm lies beneath the true subepithelial basement membrane which separates the epithelium from the lamina propria. The margins of the Rbm are marked by continuous lines. BB from S-COPD. Collagen IV antibody, x400.

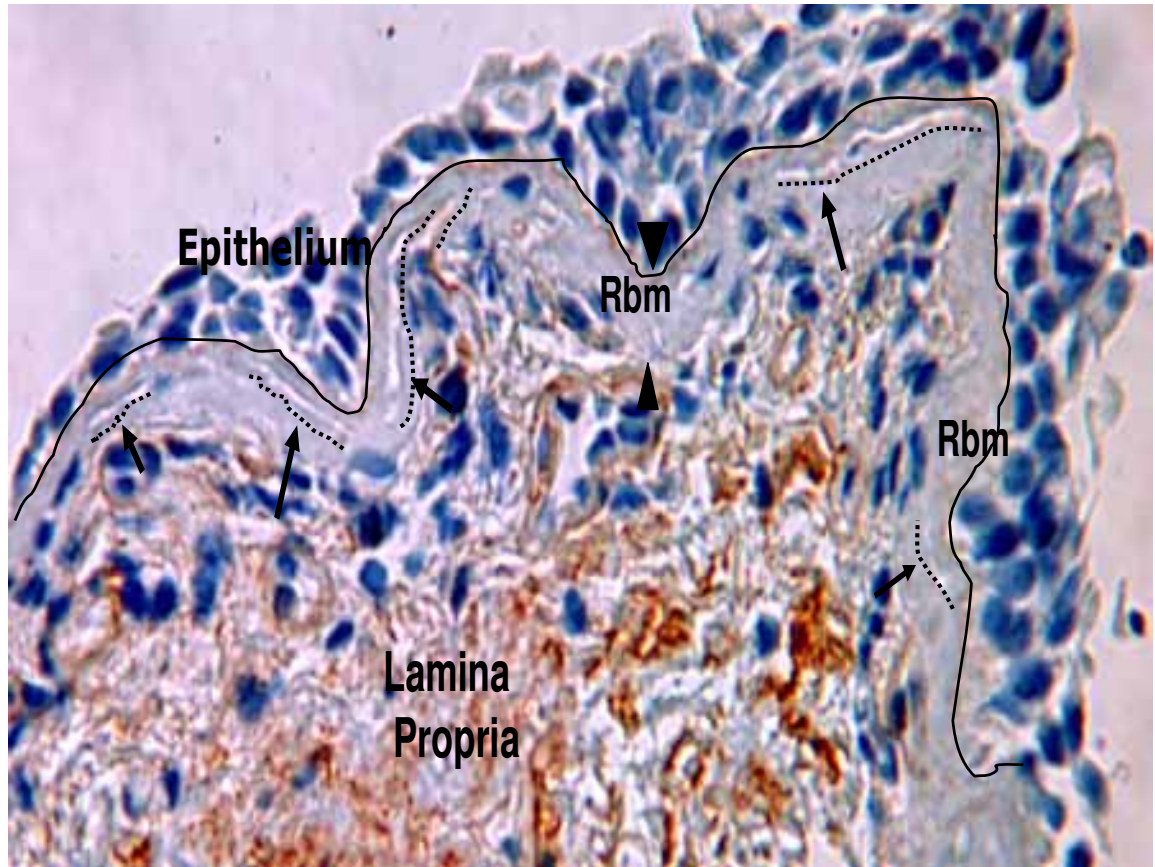
Measurements: A computer-assisted image analysis tool was used for quantifying changes in the Rbm and in the airway wall vasculature. Figure 1-1 shows the microscopic structure of a BB taken from a normal control subject.

Rbm thickness: The Rbm was identified under the epithelium and the true basement membrane (BM). Both margins of the Rbm throughout the visible microscopic field were marked (Figure 1-2). Then the average thickness of the Rbm throughout the visible field was measured with the automated image analysis tool.

Rbm morphology: The Rbm in sections generally looked non-homogenous and fragmented. This apparent fragmentation of the Rbm included pieces apparently hanging off and indeed in some sections separated from the remainder. We named these changes as “splitting” and “clefts”. We decided to use splits as a means to quantify Rbm fragmentation and compare the two groups. The length of the outer luminal margin of Rbm was measured in the whole visible microscopic field. This length was on average 2900 μm of the Rbm for eight randomly selected and examined fields in each slide. Then the length of Rbm splits was measured (Figure 1-3 A). The length of all splits was summed together and the ratio of the length of splits to the length of Rbm was calculated. Where splitting happened in parallel or overlapping layers (Figure 1-3B), all were measured separately and then added together.

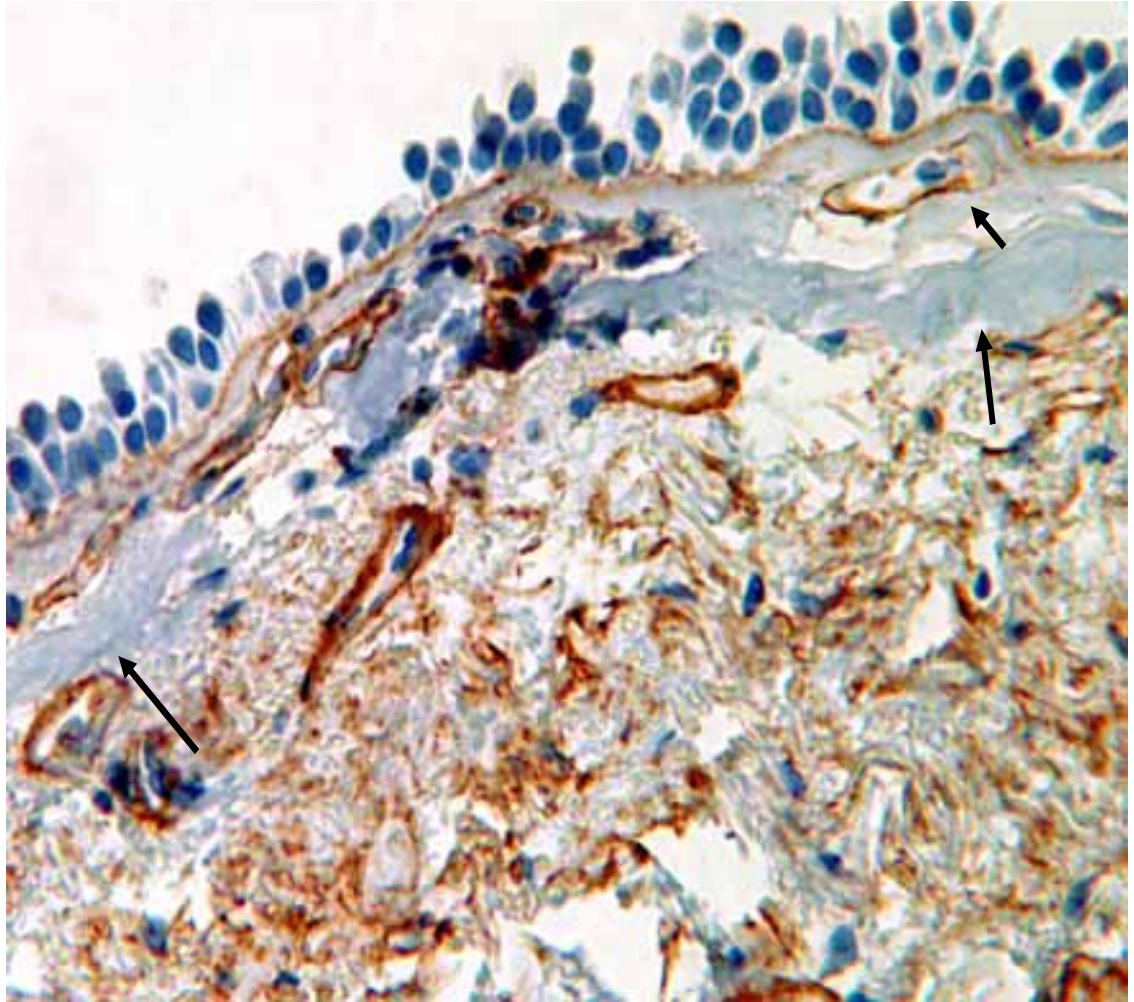
Amorphous materials: As stated, it was common in sections to see fragments of what seemed to be Rbm material separated and in the luminal part of the LP. They lay at different depths in the LP and were completely separate from the Rbm (Figure 1-4). I attempted to quantify them to compare the two groups. The length of amorphous material was measured and also divided by the length of the Rbm to normalise the data.

Vessels associated with the Rbm: Vessels were marked in outline with Collagen IV antibody to the endothelial BM and easily seen as cylindrical structures. Vessels appeared to be approaching to, “invading” into or residing within the Rbm in sections. To be included as Rbm associated vessels, they had to be either in contact with the Rbm



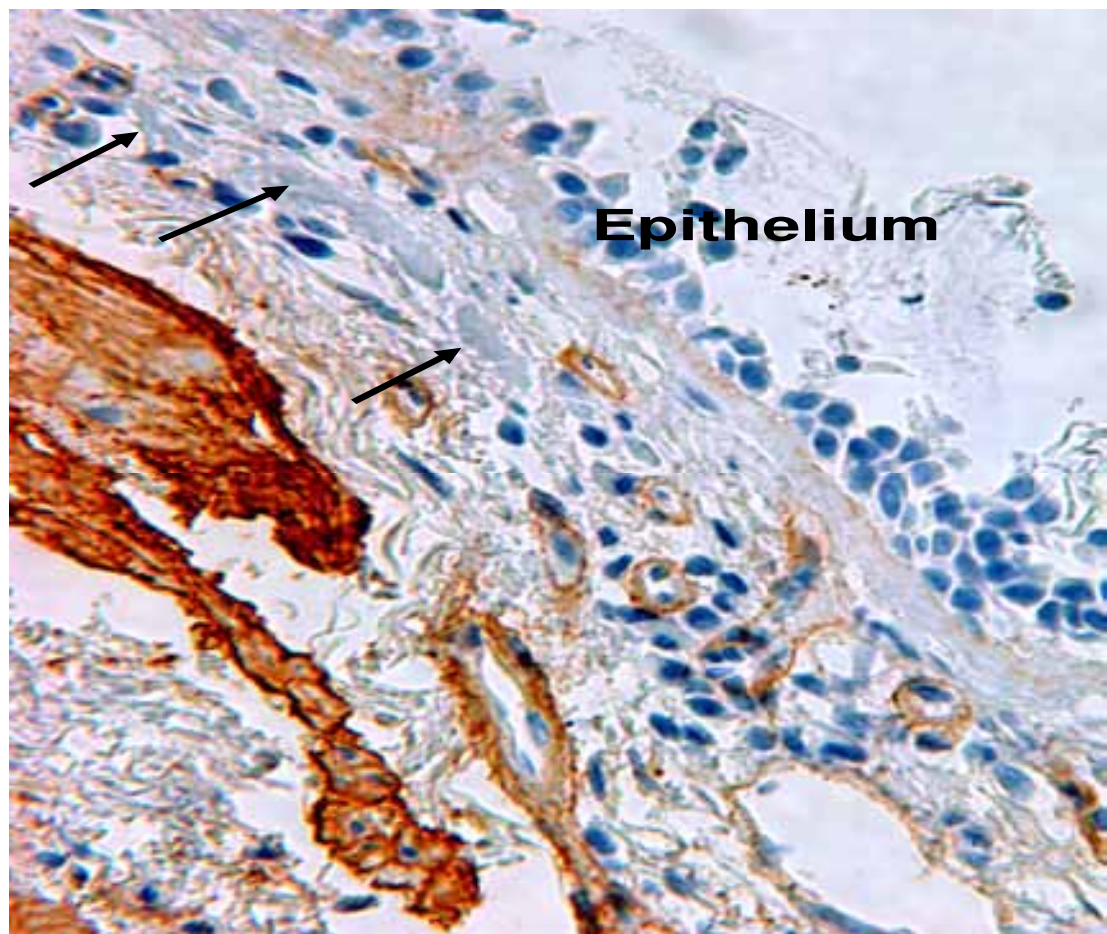
A

Figure 1-3 A. *Measuring Rbm splitting.* Rbm separates the epithelium from the lamina propria. Its margins are marked with arrowheads. The length of the outer border of the Rbm (continuous line) and splits (dotted lines and arrows) was measured. The length of splits were added together and normalized by dividing by Rbm length. Collagen IV, x400.



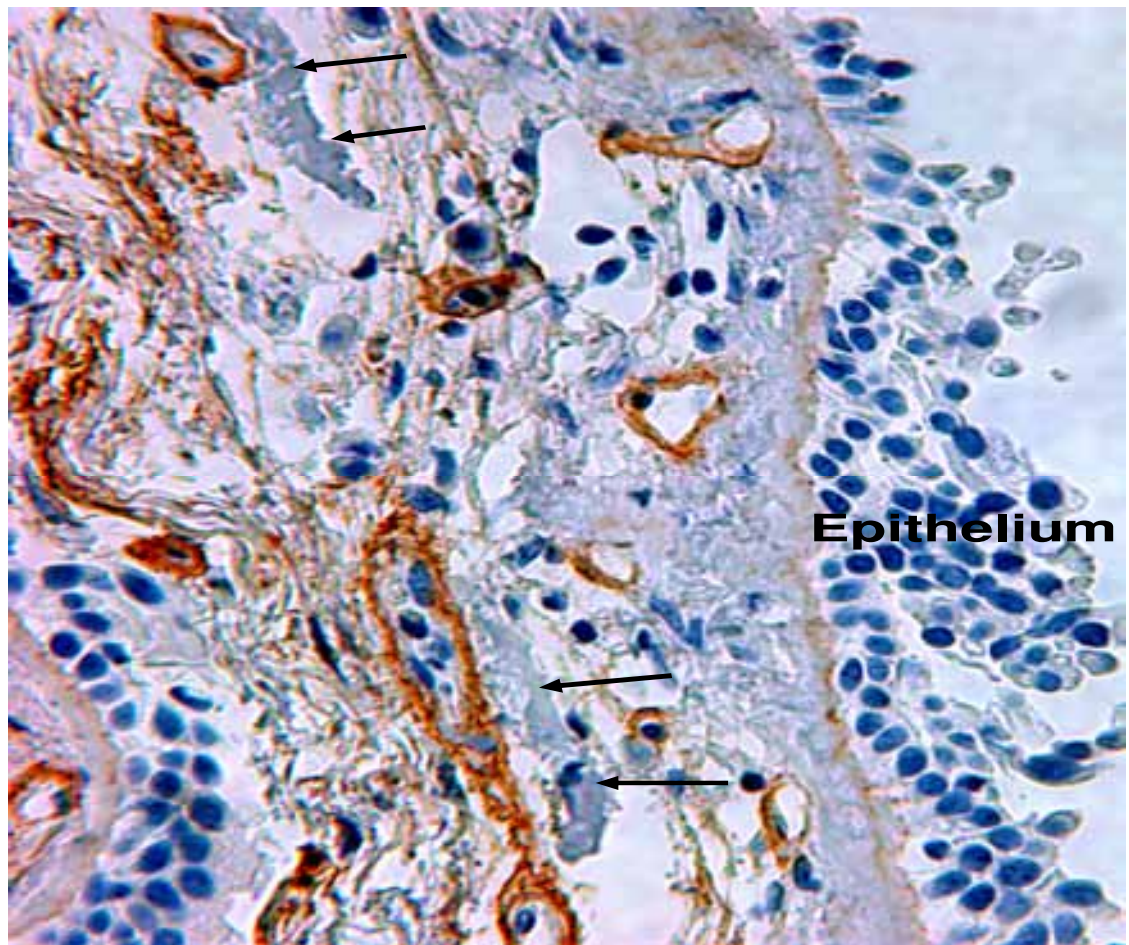
B

Figure 1-3 B. *Measuring Rbm splitting.* Arrows indicate Rbm splitting. Two parallel splits can be seen in this picture. BB from ES-COPD. Collagen IV, x400.



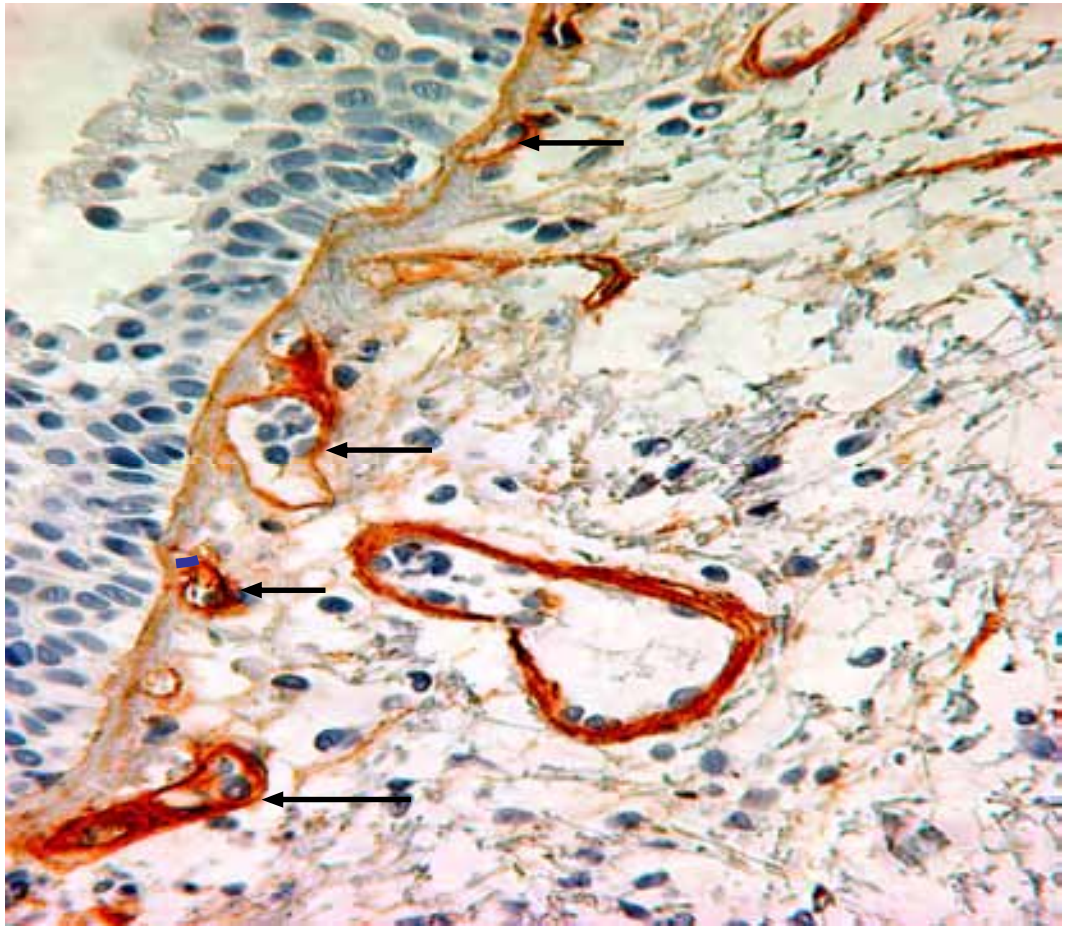
A

Figure 1-4 A. *Amorphous material* (arrows). BB from ES-COPD. Collagen IV, x400.



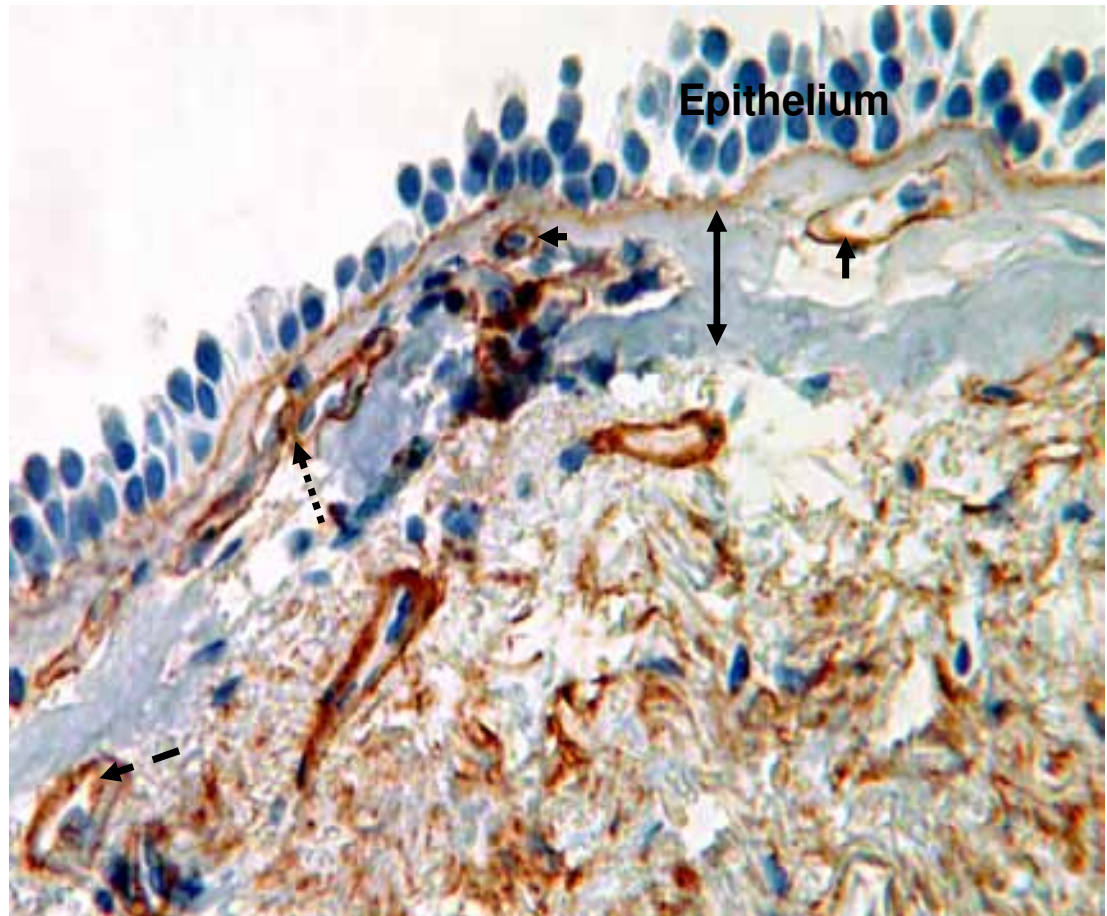
B

Figure 1-4 B. *Amorphous material* in the LP is shown with arrows. BB from ES-COPD. Collagen IV, x400.



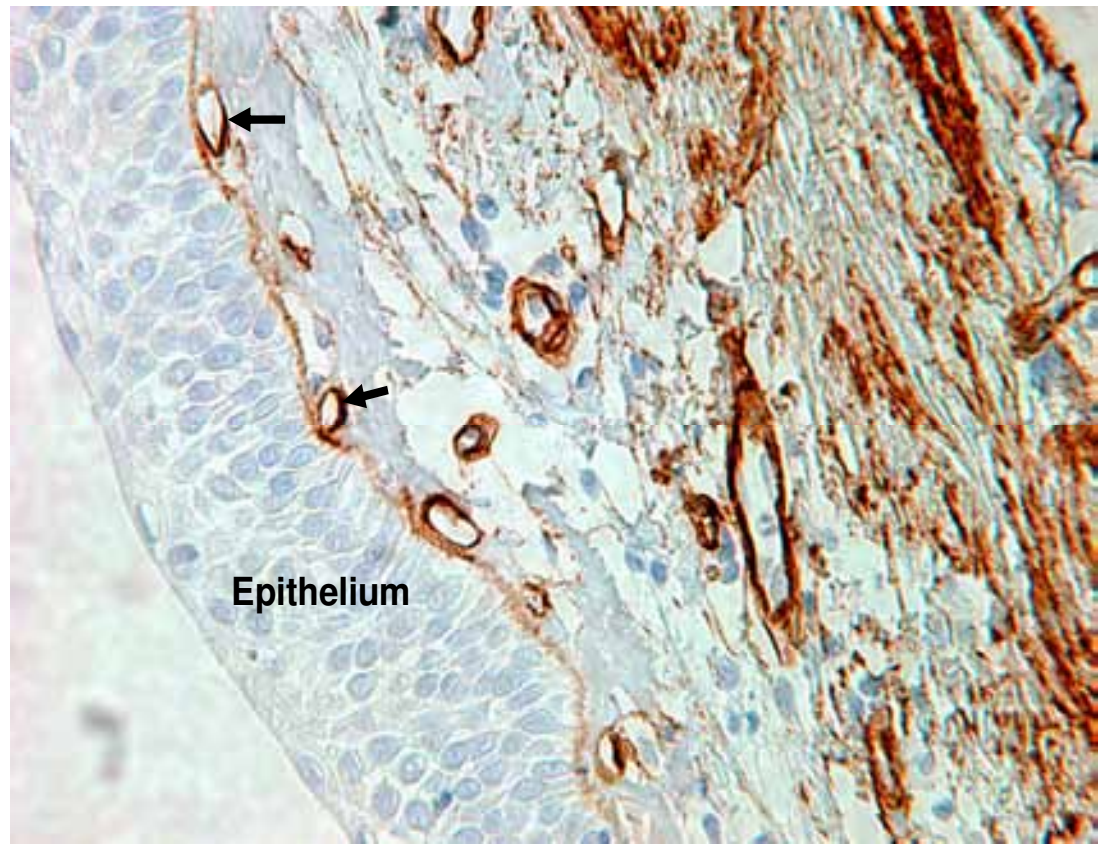
A

Figure 1-5 A. *Rbm-associated vessels.* To be included in the analysis of the Rbm-associated vessels, they needed at least to be either directly in contact with the deep margin of the Rbm (arrows) or, as seen in Figure 5 B in the next page, be embedded in the Rbm. BB from S-N. Collagen IV, x400.



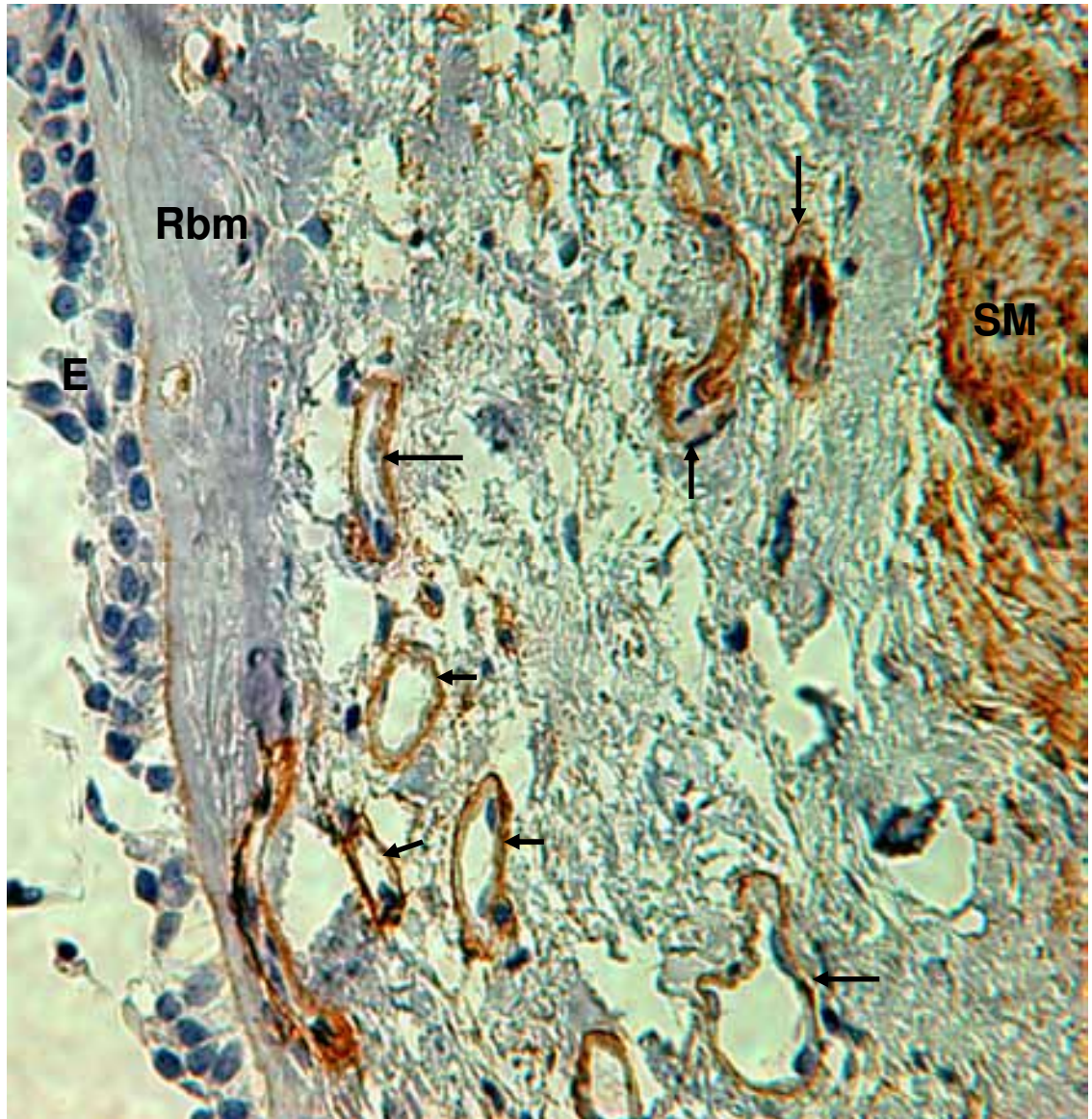
B

Figures 1-5 B. *Rbm-associated vessels.* Rbm (\updownarrow) is fragmented. Vessels are either in contact with the deep margin of the Rbm (interrupted arrow, and also Figure 5 A in the previous page) or embedded partially (dotted arrow) or completely (dense arrows) within the Rbm. BB from ES-COPD. Collagen IV, x400.



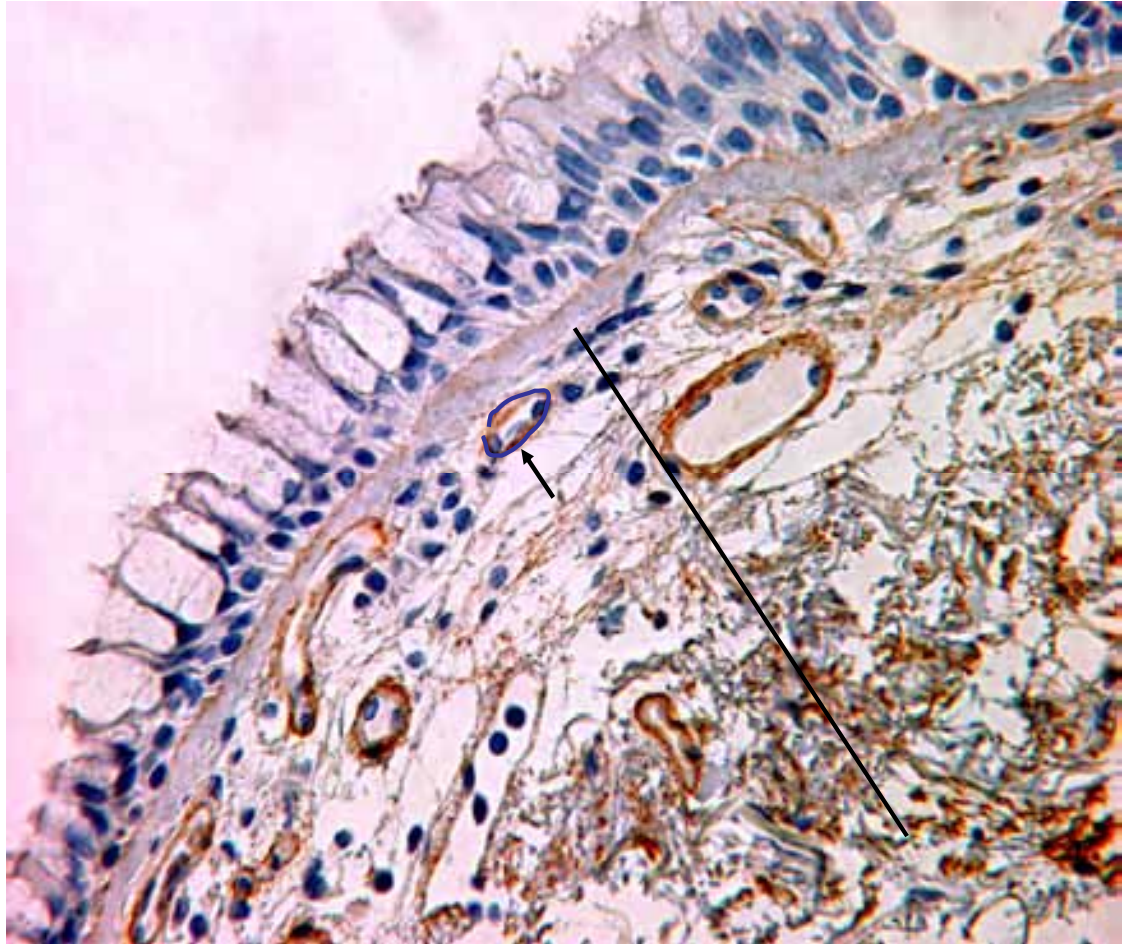
C

Figure 1-5 C. *Rbm-associated vessels.* Example of vessels embedded within the Rbm (arrows). Collagen IV, x400.



A

Figure 1-6 A. *Vessels in the LP.* Vessels (black arrows) are stained with collagen IV. BB from S-COPD. Collagen IV staining, x400.



B

Figure 1-6 B. Vessels in the LP. Vessels in the lamina propria were measured to a depth of 150 μm from the internal (deep) border of the Rbm (black straight line). Vascular area was measured as all the structures enclosed by collagen IV staining of the endothelial BM as seen here with the vessel indicated by a black arrow. BB from ES-COPD. Collagen IV staining, x400.

or embedded (partially or completely) within the Rbm (Figure 1-5). I called all of these vessels together “Rbm vessels” and I quantified their number and cross sectional area and summated them. Again, dividing by the length of the Rbm normalised the measurements.

Vessels in the LP: Vessels in the LP were marked in outline with Collagen IV antibody to the endothelial BM (Figure 1-6). The number and cross sectional areas of all the vessels to the depth of 150 μm in the LP from the internal border of the Rbm were measured. All the vessels were completely separated from the Rbm. The mucous glands and smooth muscle were excluded. Then the total number and cross sectional area of vessels were divided by the surface area of the LP for normalization, and examined to calculate vascular density and %vascular area respectively.

Statistical analyses: Normally distributed data were compared between the two groups using independent samples t-test. For non-normally distributed data Mann-Whitney test was used. Two-tailed p values less than 0.05 were considered as statistically significant. Data are reported as mean (SD) or mean (range) for normally distributed variables and as median (range) for variables with non-normal distribution.

Results and preliminary discussion:

For demographics of the population in this preliminary study please see Table 1-1.

Table 1-2 summarises the anatomical/pathological data from the study.

S-COPD subjects were significantly older than normal controls [median (range) 60.3 (48-69) vs. 48.4 (20-68), $p < 0.03$]. As expected from our recruitment criteria, pack year smoking history, FEV1% predicted and FER were significantly different between groups ($p < 0.001$ for all).

Rbm thickness in S-COPD was not different from controls [median (range) 6.4 (2.7-13.7) vs. 5.2 (2.3-9.6) μm , $p = 0.2$]. Length of splitting ($\mu\text{m}/\mu\text{m}$ of Rbm length x 100) was significantly greater in the S-COPD group compared to the H-N group [median

Table 1-1. Demographics of subjects in preliminary study

Groups (numbers)	H-N (n=13)	S-COPD (n=14)	P value
Age, mean (range)	48 (20-68)	60 (48-69)	<0.03*
Gender female/male	8/5	5/9	0.2 (NS) [‡]
Pack-year smoking history, median (range)	0 (0)	47 (32-82)	<0.001 [†]
FEV1% predicted, mean (SD)	111 (12)	77 (11)	<0.001*
FER, mean (SD)	81 (71-88)	56 (46-68)	<0.001*

*t-test, [†] Mann-Whitney U test, [‡] Chi-square test, NS = not significant

Table 1-2. Summary of results for Rbm and vessels in preliminary study

Groups (numbers)	H-N (n=13)	S-COPD (n=14)	P value [‡]
Rbm thickness (μm), median (range)	5.2 (2.3-9.6)	6.4 (2.7-13.7)	0.2 (NS) [†]
Length of splitting/Rbm length x 100, median (range)	5.3 (0.0-21.4)	20.1 (0.4-42.8)	<0.01 [†]
Length of amorphous material x 100, median (range)	0 (0.0-10.9)	1 (0.0-6.4)	0.4 (NS) [†]
No. of Rbm vessels/mm Rbm, median (range)	4.5 (0.0-26.4)	9.1 (1.6-23.0)	<0.05 [†]
Area of Rbm vessels μm ² /mm Rbm, median (range)	462 (0-3263)	953 (115-2159)	0.06 (NS) [†]
No. of LP vessels No./mm ² LP, mean(SD)	353 (108)	211 (130)	<0.01*
Area of LP vessels μm ² /μm ² LP x100, mean (SD)	6.9 (2.8)	4.6 (2.1)	<0.05*

*t-test, [†] Mann-Whitney U test, [‡]NS = not significant

(range) 20.1 (0.4-42.8) vs. 5.3 (0.0-21.4), $p < 0.01$]. Length of amorphous material was not significantly different between groups.

The number of Rbm vessels was significantly higher in the S-COPD group compared to H-N [median (range) 9.1 (1.6-23) vs. 4.5 (0.0-26.4) number/mm of the Rbm, $p < 0.05$]. The area of Rbm vessels ($\mu\text{m}^2/\text{mm}$ of the Rbm) in S-COPD was not significantly different from H-N [median (range) 935 (115-2159) vs. 462 (0-3263), $p = 0.06$].

The number of vessels in the LP (number/ mm^2 of the LP) was significantly lower in the S-COPD group compared to controls [mean (SD) 211 (130) vs. 353 (108), $p < 0.01$]. The area of LP vessels ($\mu\text{m}^2/\mu\text{m}^2 \text{ LP} \times 100$) was significantly lower in S-COPD compared to H-N [mean (SD) 4.6 (2.1) vs. 6.9 (2.8), $p < 0.05$].

Our preliminary study revealed quite novel findings in the biopsies from airway in the currently smoking COPD subjects. It showed that compared to the control group, in S-COPD:

- The Rbm is highly fragmented.
- The Rbm is hypervascular.
- And the LP is hypovascular.

These findings will be discussed in the comprehensive study in chapter five.

The origin, structure and biological significance of amorphous material remains unclear.

Table 1-1 shows there are two important and potentially confounding differences between the control and COPD groups. First, the control group was significantly younger, but we did not find any relationship between our findings and age (see Chapter Five for more details). Second, we are unable to distinguish a smoking effect from the effect of COPD as a disease in causing these changes. We have therefore set out hypothesis that the changes I have described are due specifically to COPD. To test this hypothesis, I decided to repeat the study with more subjects to include those who smoke

but have normal lung function (S-N) and subjects who have COPD but are ex-smokers (ES-COPD) and carry out a comprehensive cross-sectional analysis (Chapter Five).

Another question raised from the preliminary study was: *what is the reason for the pattern of vessel distribution in COPD group?* As an attempt to answer this question, I have assessed our samples for VEGF and TGF- β , two central angiogenic growth factors.

Preliminary study: Angiogenic factors in COPD

Introduction:

With our findings on airway remodelling in the pilot study (see above and table 1-2) I have assessed the activities of two specific angiogenic factors to see if they contribute to changes in the distribution of vessels in the Rbm and LP.

Research question:

Can vascular alterations of the Rbm and LP in the S-COPD group be explained by the changes in expression and stainability of angiogenic factors, VEGF and TGF- β ?

Material and methods:

For details of material and methods please see chapter three.

This was a cross-sectional study, done in the same coded and blinded way described above.

Subjects: The same tissue samples used for the previous part of the preliminary study were used for this work (see above).

Measurements:

Vascular endothelial growth factor (VEGF): Using a computer-assisted image analysis tool (see Chapter Three for details) we assessed our samples for VEGF.

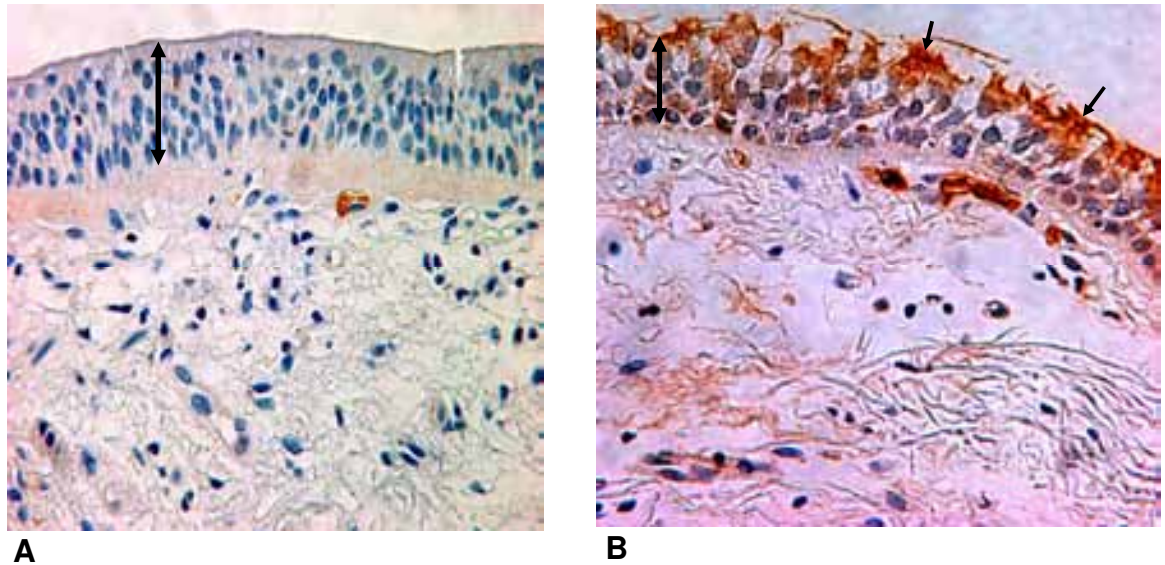


Figure 1-7. *Epithelial VEGF staining.* The epithelium in **A** has almost no VEGF staining and in **B** is stained strongly for VEGF (arrows). The thickness of the epithelium is shown by two headed arrows. BB from H-N (A) and S-N (B). VEGF staining, x400.

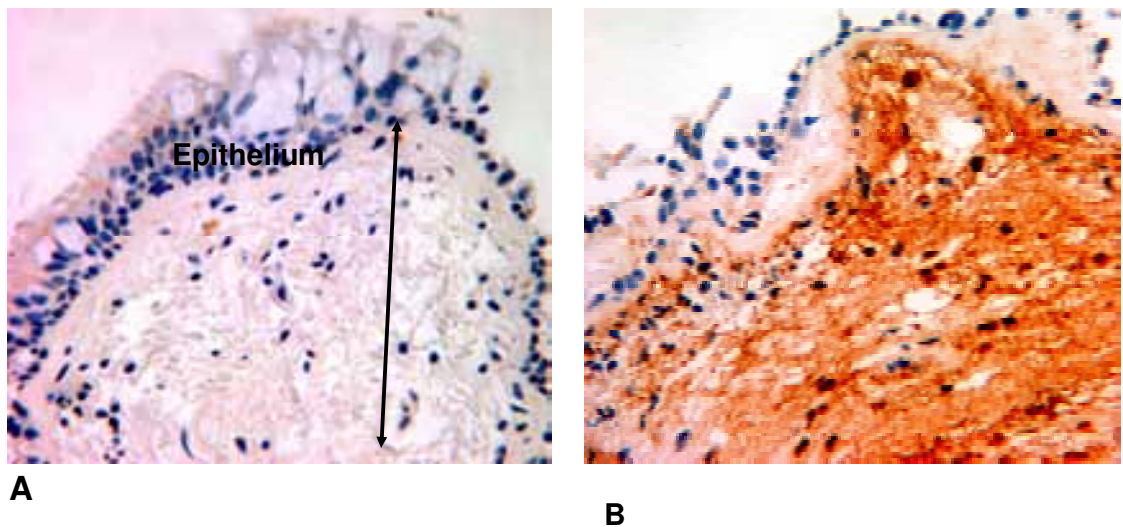


Figure 1-8. *VEGF staining of the LP.* The epithelium is marked in **A**. The surface area of the LP was measured to the depth of 150 µm (two headed arrow) and by using automated software, percent staining of VEGF was measured in this area of interest. VEGF staining of the LP in **A** is much lighter than **B**. BB from ES-COPD. VEGF staining, x400.

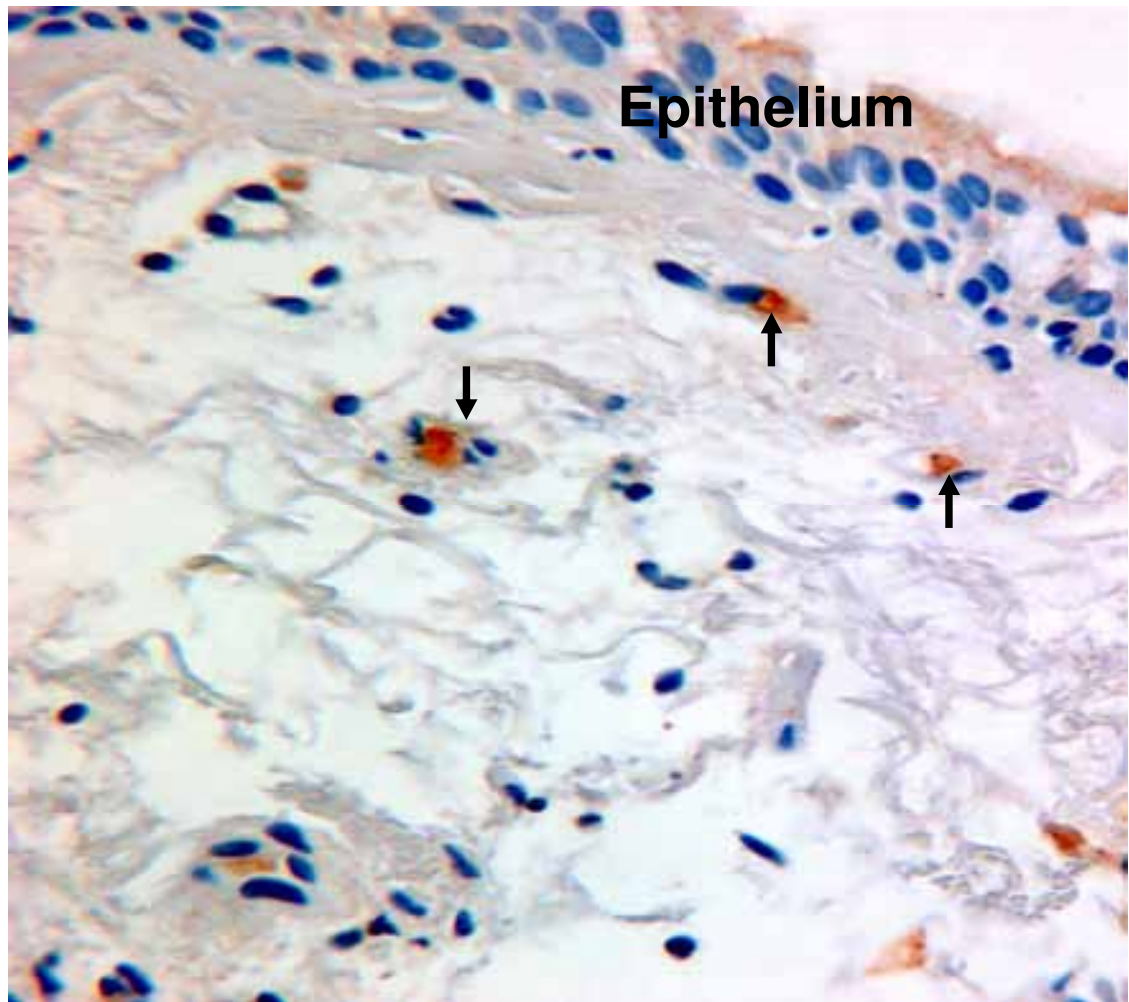
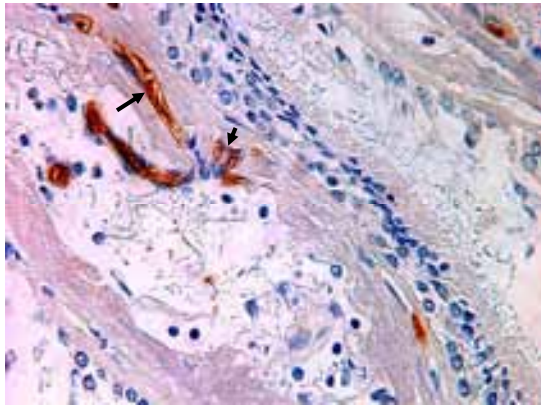
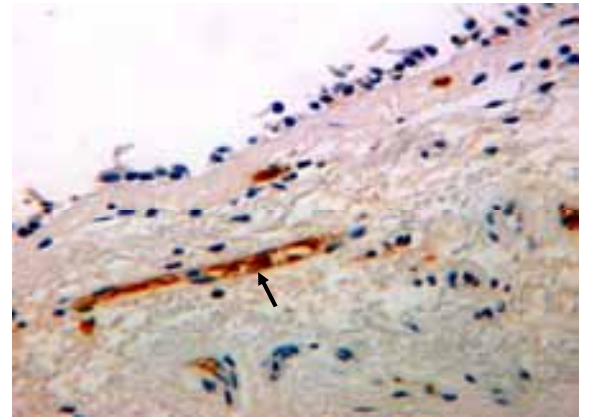


Figure 1-9. *Cells stained for VEGF in the LP. The cells are shown with arrows. VEGF staining, x400.*

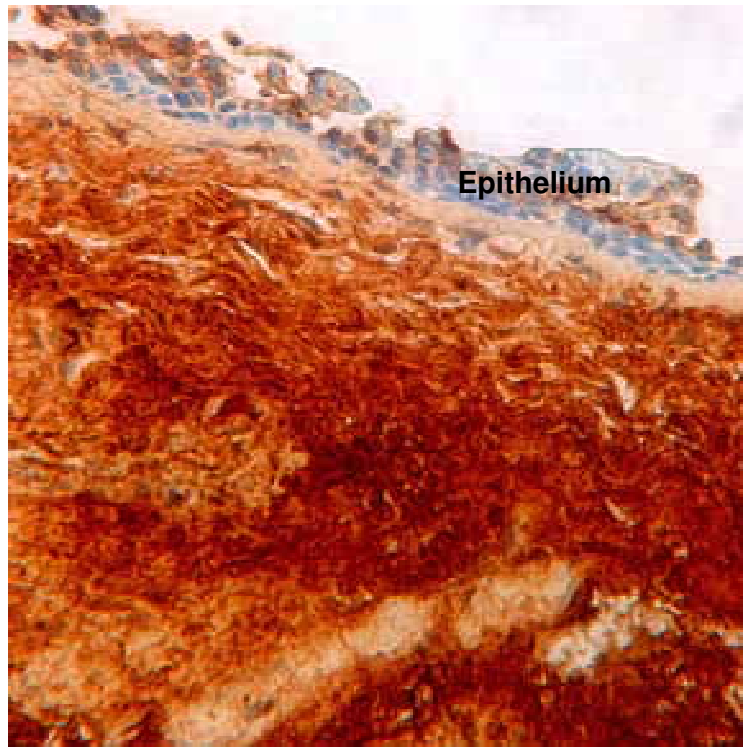


A



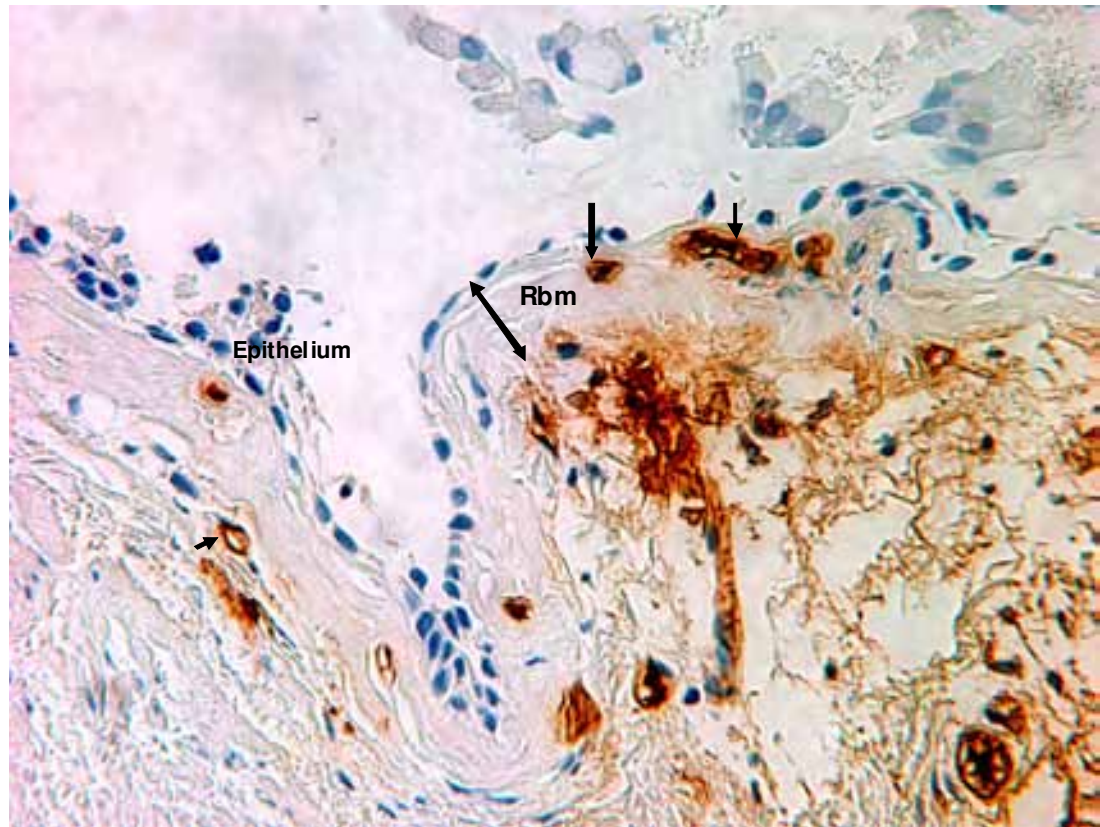
B

Figure 1-10. *Vessels stained with VEGF in the Rbm (A) and LP (B). Arrows show the vessels. BB from ES-COPD. VEGF staining, x400.*



A

Figure 1-11 A. *TGF- β staining of tissue.* Borders of the tissue are obscured and it is impossible to recognize different components in the LP. Epithelial staining can be measured. BB from S-COPD. TGF- β 1 staining, x400.



B

Figure 1-11 B. *TGF- β staining of tissue.* TGF- β stained vessels in the Rbm (arrows). This tissue is exceptionally clear in the LP. The width of the Rbm is shown by two-headed arrow. BB from S-COPD. TGF- β 1 staining, x400.

Percent area of the epithelium, Rbm and LP stained for VEGF was measured using the automatic software (Figures 1-7 and 1-8). The area of interest was manually selected assisted by the software. The whole visible area of the epithelium, Rbm and LP to the depth of 150 μm was selected. The tissue stained with VEGF antibody was defined to minimise nonspecific background staining. In summary, the software was set as sensitivity of 4, Drop Colours of 1 pixel, and Expand Selection of 1 colour index. These settings remained consistent throughout analysis of all slides, and seemed empirically to be a good robust solution. Then the percentage surface area of the selected area that was stained by VEGF was calculated automatically. The number of cells positive for VEGF was counted in the Rbm and LP to the depth of 150 μm (there was no visible cellular infiltration in the epithelium) (Figure 1-9). The number and cross sectional area of the vessels positive for VEGF in the Rbm and LP to the depth of 150 μm were also measured (there were no visible vessels in the epithelium) (Figure 1-10).

TGF- β 1: This preliminary study was carried out in collaboration with another PhD student, Mr. S.S. Sohal, who is working primarily in our group on epithelial-mesenchymal transition (EMT) in smoking and COPD. Most samples showed generalised strong staining of TGF- β 1 despite substantial dilution (1/16,000) of the antibody. The stain remained so dark that it obscured the natural borders of the tissues and its components (Figure 1-11A). Therefore, measurement of vessels and cells in the LP could not be achieved in most samples. It was only possible to measure epithelial staining of TGF- β 1 (Figure 1-11A) and the number of vessels stained for TGF- β 1 in the Rbm (Figure 1-11B). Even then, some slides with especially heavy TGF- β 1 background staining could not be used.

Results:

Demographics were shown in Table 1-1.

VEGF: Table 1-3 summarises the results for VEGF measurements in the two groups. Briefly, there were no difference between the S-COPD and H-N groups in percent area stained for VEGF in the epithelium [median (range) 0.2 (0.0-6.1) vs. 0.8 (0.0-19.5), $p =$

Table 1-3. Summary of results for VEGF in preliminary study[†]

Groups (numbers)	H-N (n=13)	S-COPD (n=14)	P value*
Epithelial VEGF percent area staining	0.8 (0.0-19.5)	0.2 (0.0-6.1)	0.2 (NS)
Rbm VEGF percent area staining	0.0 (0.0-0.1)	0.0 (0.0-0.1)	0.2 (NS)
LP VEGF percent area staining	3.9 (1.2-50.2)	3.0 (0.3-9.9)	0.2 (NS)
No. of vessels in the Rbm stained for VEGF/mm Rbm	0.0 (0.0-10)	1.4 (0.0-6.9)	<0.005
Area of vessels in the Rbm stained for VEGF $\mu\text{m}^2/\text{mm Rbm}$	0.0 (0.0-1115)	94 (0.0-746)	<0.005
Percent of No. of Rbm vessels stained for VEGF/mm Rbm x 100	0 (0-34)	22 (0-92)	<0.005
Percent of area of Rbm vessels stained for VEGF/mm Rbm x 100	0 (0-34)	18 (0-99)	<0.005
No. of vessels in the LP stained for VEGF/ mm^2 LP	93 (0-423)	124 (17-246)	0.5 (NS)
Area of vessels in the LP stained for VEGF $\mu\text{m}^2/\mu\text{m}^2$ LPx100	1.2 (0.0-5.7)	1.1 (0.2-4.4)	0.7 (NS)
No. of cells positive for VEGF in the LP/ mm^2 LP	169 (25-482)	75 (6-369)	0.2 (NS)

*Mann-Whitney U test, NS = not significant[†] All values are median (range)

0.2], Rbm [median (range) 0.0 (0.0-0.1) vs. 0.0 (0.0-0.1), $p = 0.2$] and LP [median (range) 3.0 (0.3-9.9) vs. 3.9 (1.2-50.2), $p = 0.2$]. Neither were there differences in the number of vessels stained for VEGF [median (range) 124 (17-246) vs. 93 (0-423) number/mm² LP, $p = 0.5$], area of vessels stained for VEGF [median (range) 1.1 (0.2-4.4) vs. 1.2 (0.0-5.7) $\mu\text{m}^2/\mu\text{m}^2$ LPx100, $p = 0.7$] and number of cells stained for VEGF [median (range) 75 (6-369) vs. 169 (25-482) number/mm² LP, $p = 0.2$] in the LP between S-COPD and controls.

There were very few cells positive for VEGF in the Rbm and so were not formally compared between groups. However, vessels in the Rbm were significantly different between the study groups. The number [median (range) 1.4 (0.0-6.9) vs. 0.0 (0.0-10) number/mm Rbm, $p < 0.005$] and area of vessels [median (range) 94 (0-746) vs. 0 (0-1115) $\mu\text{m}^2/\text{mm}$ Rbm, $p < 0.005$] in the Rbm stained for VEGF were higher in the S-COPD group compared to the control group. Percent of number [median (range) 22 (0-92) vs. 0 (0-34), $p < 0.005$] and area [median (range) 18 (0-99) vs. 0 (0-34), $p < 0.005$] of vessels stained for VEGF (the number and area of vessels stained for VEGF divided by total number and area of vessels stained with Collagen IV antibody x 100) in the Rbm were higher in S-COPD compared to H-N.

TGF- β : *TGF- β* : Percent area of the epithelium stained for TGF- β 1 was not significantly different between the two groups; but number [median (range) 3.4 (0.0-8.12) vs. 0.0 (0.0-7.0), $p < 0.005$] and area [median (range) 324 (0-2882) vs. 0 (0-545), $p < 0.005$] of Rbm vessels stained for TGF- β 1 were significantly higher in S-COPD versus H-N. Therefore, I only counted Rbm vessels in my subsequent extended study and data are presented later.

Discussion:

Higher vessel number and vessel area stained for VEGF in the Rbm showed that vessel-associated VEGF activity may be higher in this compartment. We calculated the percent of vessels stained for VEGF in the Rbm (Table 1-3) to be sure that this difference is not simply the consequence of hypervascularity of the Rbm in the S-COPD group. On the

Table 1-4. Summary of published studies on TGF- β in COPD and smokers

Study group	Methods	Main results
(Zanini et al., 2009)	BB from: -10 ex-smokers with moderate to severe COPD (group 1) -10 ex-smokers with moderate to severe COPD on ICS (group 2) -8 control subjects (CS) with pulmonary nodules or haemoptysis	1.Number of TGF- β + cells in the LP was higher in group 1 c.f. CS
(Zandvoort et al., 2006)	Lung tissue obtained for various reasons from: -11 moderate COPD with no chronic bronchitis (group 1) -8 very severe COPD with no chronic bronchitis (group 2) -8 control subjects (CS) -all of group 2, 5 subjects in group 1 and 3 in CS were ex smokers	1.TGF- β 1 reduced in airway epithelial cells, stromal cells and type II alveolar cells in group 1 c.f. CS 2.Current smokers had higher TGF- β 1 in airway epithelial cells compared to ex-/non-smokers
(Takizawa et al., 2001)	Ultrathin-bronchoscopic brushing of bronchiolar epithelial cells from: -17 COPD -18 current smokers without COPD -15 nonsmokers without COPD	1.Higher TGF- β expression in small airway epithelial cells in smokers and in COPD compared to CS
(de Boer et al., 1998)	Lung tissue from: -14 current-/ex-smokers with COPD -14 current-/ex-smokers without COPD -all had peripheral lung cancer	1.Higher TGF- β 1 in bronchiolar and alveolar epithelium in COPD
(Vignola et al., 1997)	BB from: -19 chronic bronchitis with or without COPD -24 asthmatics -13 control subjects (CS)	1. TGF- β higher in the epithelium and the LP in chronic bronchitis and asthma c.f. CS

other hand, hypovascularity of the LP was not accompanied by lower VEGF content in this compartment. In the comprehensive study, I included S-N and ES-COPD to determine if our findings were smoking effects or specifically related to COPD itself.

There are at least 12 angiogenic factors described in the literature (see page 64, Chapter Two). I decided to study VEGF and TGF- β because they are the best known and most studied mediators of angiogenesis, and most likely to be implicated in a chronic inflammatory condition such as COPD.

We were not able reasonably to assess TGF- β 1 content of tissue compartments except for the epithelium in our samples because of very heavy and uniform background staining. Some authors have reported on TGF- β 1 in smokers and COPD subjects (Table 1-4) without commentary on the inherent difficulties we have encountered. No one, to our knowledge, has ever counted Rbm vessels positive for TGF- β 1 and I believe the compartmentalisation of the mucosa in smokers and COPD as I have done is quite novel. Two of the previous studies used BB like us (Vignola et al., 1998) (Zanini et al., 2009). Zanini et al. found higher TGF- β positive cells in the LP in BB from moderate to severe COPD patients compared to control subjects but without more relevant groups. Interestingly, they reported using antibody with only 1:25 dilution to stain tissue for TGF- β . However, despite diluting the antibody up to 1:16000, we found it impossible to recognise cells containing TGF- β 1 in many of our samples because the LP was diffusely and darkly stained (Figure 1-11A). Vignola et al. found higher TGF- β 1 in the epithelium and the LP of patients with “bronchitis” and asthma compared with normal control subjects. The figures they provided looked very different from ours; probably differences in method of immunostaining or source of antibodies explain these differences. We did not find higher TGF- β in the epithelium of COPD subjects, but they did not have chronic bronchitis as a diagnosis. To overcome the problems of non-specific or ubiquitous staining for TGF- β , I believe that future works should utilize molecular methods, e.g. RT-PCR, rather than immuno-chemical staining to evaluate TGF- β 1 expression in the LP in our subjects. However, this has the inherent problem of being unable to assess actual protein production or what cells are producing the protein.

Further, if cell populations differ between groups then this problem is greatly compounded.

With the background results from our preliminary studies I again hypothesised that these changes are specific for COPD. Thus, the *hypotheses* that were developed from my preliminary study were:

In the next comprehensive, multi-group analysis:

1. Rbm splitting and vascular changes in the Rbm and LP are specific for COPD.
2. These changes may be related to changes in VEGF activity.
3. These changes may be related to changes in TGF- β 1 activity.

To test this hypothesis, I included two new groups, smokers with normal lung function (S-N) and ex-smokers with COPD (ES-COPD) in addition to the previous samples to distinguish the effects of smoking from the pathology of COPD in terms of airway remodelling. All assessments were repeated de novo, and slides assessed randomly and blinded to source and diagnosis.

Longitudinal study:

As inhaled corticosteroids (ICS) have been shown to be effective in airway remodelling in asthma (Olivieri et al., 1997) (Orsida et al., 1999) (B. N. Feltis et al., 2007) and are commonly used in clinical practice for COPD (GOLD, 2007) (B. R. Celli et al., 2004), I also decided to evaluate the effects of treatment on these manifestations of airway remodelling in the BB taken previously from our COPD patients.

For this **longitudinal study** my **aims** were:

1. To study the effects of ICS on airway remodelling in BB from COPD; i.e.:
 - Effects of ICS on Rbm remodelling I described in the preliminary study
 - Effects of ICS on vascular remodelling
 - Effects of ICS on angiogenic factors

Chapter Two

Literature Review and Background

Chronic Obstructive Pulmonary Disease (COPD):

Summary:

COPD is a common disease and a worldwide health problem which consumes huge amount of the health budget in developed countries including Australia. Smoking is the most important cause of COPD worldwide, though in developing countries indoor air pollution has a big impact on women.

In this chapter, overviews of *definition, aetiology, pathology, pathogenesis, diagnosis* and *management* of COPD are presented. The parts of the literature that are most relevant to my project especially *airway remodelling* and *angiogenesis* are emphasised.

In *Pathology and Pathogenesis* I describe how COPD is a complex disease potentially involving lung parenchyma, small airways and large airways. Pulmonary inflammatory changes occur in all smokers in the early stages before airflow obstruction begins. COPD evolves in a subgroup of subjects who smoke. After explaining classical

pathological changes of emphysema and chronic bronchitis, newer studies on airway wall *remodelling* are described in details with emphasis on *vascularity* and *reticular basement membrane (Rbm)* changes. Then the *physiological* relevance of these changes is discussed.

In contrast to asthma, published investigations using per-bronchoscopic bronchial biopsies (BB) to study airway remodelling in COPD are few. There is recognized substantial need for more work on this subject.

The Rbm has been reported to have the same thickness as control groups in COPD, in contrast to asthma where it is classically thickened. The number and area of vessels in the airway wall have been variably reported to be higher or the same as in control groups in different studies. Reported sputum *VEGF* levels have varied with the morphology of COPD; i.e. it has been reported to be increased in chronic bronchitis, reduced in emphysema and the same level as normal controls in mixed type of COPD.

The inter-relationship between inflammation, epithelial-mesenchymal transition (EMT) and angiogenesis is an important subject very relevant to my project. To address it, I needed to cite and discuss recent publications about intracellular mechanisms of inflammation.

Glucocorticoids are commonly used in COPD; but their mechanisms of action are not completely clear. We know that they are not as effective in COPD as they are in asthma. Recent studies about glucocorticoids action and resistance to them are discussed.

1-Definition:

According to the pre-eminent Global Initiative for Obstructive Lung Disease (GOLD) COPD is a preventable and treatable disease characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases (GOLD, 2007), most commonly cigarette smoke constituents. By this definition the GOLD committee tried to modulate the opinion of clinicians and health workers towards a more optimistic view about COPD. Treatment with inhaled corticosteroids (ICS) and/or long-acting bronchodilators (LABA) has been shown to improve health status and lung function parameters and to reduce the severity of exacerbations and inflammation (P.M.A. Calverley et al., 2006; D. W. Reid et al., 2008). However, in reality, clinicians know that, at least in its major clinical and physiological manifestations, treatment may only slow the progression of COPD but the disease is nonreversible (P. J. Barnes, 2009).

COPD is clinically and pathologically classified as *emphysema* or *chronic obstructive bronchitis* (GOLD, 2007) or some combination of the two. Thus patients with COPD may present as mainly emphysematous, mainly bronchitic or a combination of both.

Destruction of alveolar septae and formation of large air spaces is called emphysema (*Terminology, Definitions, and Classification of Chronic Pulmonary Emphysema and Related Conditions: A REPORT OF THE CONCLUSIONS OF A CIBA GUEST SYMPOSIUM*, 1959) (see Pathology). “Simple” chronic bronchitis is defined as productive cough for at least 3 months each year in two consecutive years, in the absence of other pulmonary disorders (S. D. Shapiro, Snider Gordon L., Rennard Stephen I., 2005). (This definition was coined in the 1950s for mainly epidemiological purposes). At the same time some patients have airway obstruction; this is called chronic obstructive bronchitis (which is not “simple”). The nomenclature is therefore somewhat complex and can be confusing. In reality, there is obstructive airway disease, cough/sputum and emphysema and each can occur alone or in any combination.

1.a-Airflow limitation (airflow obstruction):

Airflow obstruction is determined by lung function tests (Figure 2-1). Spirometry can measure lung volumes and flow rates. The vital capacity (VC) is the volume of the lung that you can exhale out after a deep and complete inhalation. The forced vital capacity (FVC) is the same as the VC when done as forcefully as possible. The volume exhaled in the first second of exhalation is called the forced expiratory volume in one second (FEV1). When less than 70% of FVC can be exhaled in the first second of forceful exhalation [FEV1/FVC ratio, or forced expiratory ratio (FER) less than 70%] airflow limitation is diagnosed. In COPD FER is equal or less than 69% and classically does not increase to 70% or more with acute dose of bronchodilators or long term treatment with corticosteroids (GOLD, 2007) (Pauwels, Buist, Calverley, Jenkins, & Hurd, 2001).

Bronchodilator responsiveness (BDR) is used as a means to distinguish COPD from asthma. Asthma is an obstructive airway disease which is “classically” characterised by reversibility of airway obstruction, defined as 15% or more increase in FEV1 after bronchodilator inhalation. Even so, many patients with a history of asthma have some degree of “fixed” airflow obstruction, and this can be a major feature. COPD is classically considered as non-responsive to bronchodilators, despite the fact that there is about 8% increase in post-bronchodilator FEV1 on average in this population and much more in many subjects. Therefore, many subjects with COPD and no history of asthma, have at least some degree of BDR, usually less than 15%, but some are in the traditional asthma range with BDR>15% (D. W. Reid et al., 2003). This is yet another confusing aspect of the definition and diagnostic algorithm.

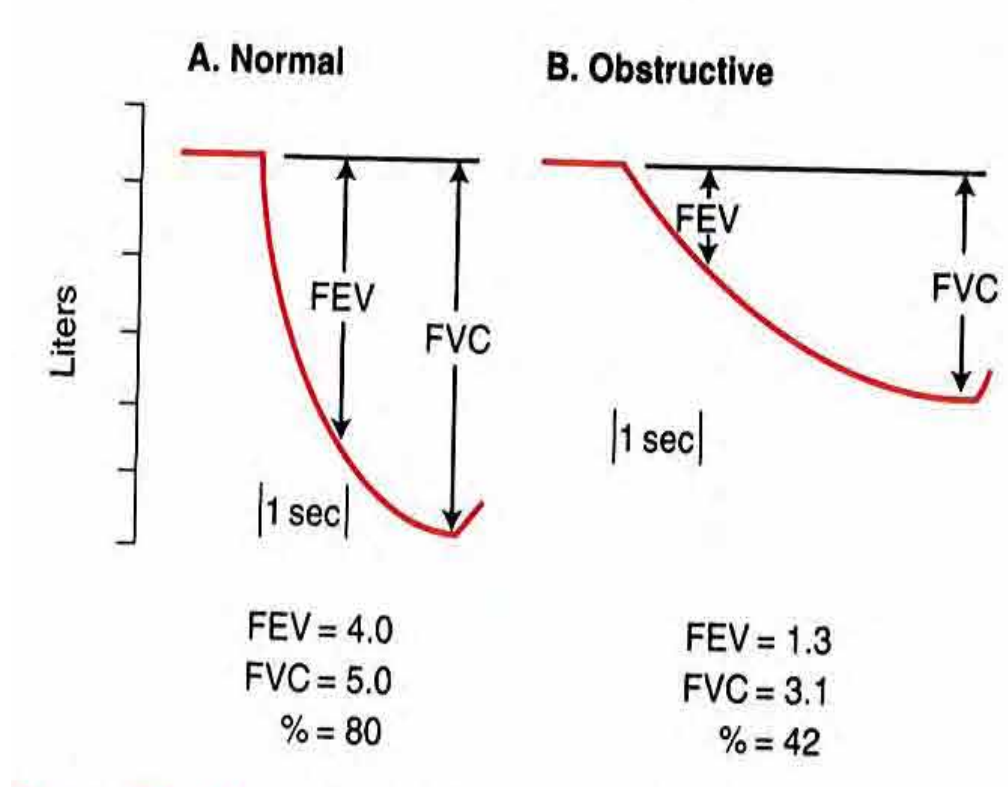


Figure 2-1. *Spirometry comparing normal pattern with obstructive pattern.* (Source: (West, 2003))

1.b-Classification:

COPD is classified by severity of airflow limitation in spirometry.

Classification of severity of COPD by spirometry:

Stage I: Mild	FEV ₁ /FVC < 0.70 FEV ₁ ≥ 80% predicted
Stage II: Moderate	FEV ₁ /FVC < 0.70 50% ≤ FEV ₁ < 80% predicted
Stage III: Severe	FEV ₁ /FVC < 0.70 30% ≤ FEV ₁ < 50% predicted
Stage IV: Very Severe	FEV ₁ /FVC < 0.70 FEV ₁ < 30% predicted or FEV ₁ < 50% predicted plus chronic respiratory failure

FEV₁: forced expiratory volume in one second; FVC: forced vital capacity; respiratory failure: arterial partial pressure of oxygen (PaO₂) less than 8.0 kPa (60 mm Hg) with or without arterial partial pressure of CO₂ (PaCO₂) greater than 6.7 kPa (50 mm Hg) while breathing air at sea level.

(Source: (GOLD, 2007)).

2-Epidemiology:

The prevalence of COPD rises with age and is more than 10% in those older than 40 years (Halbert et al., 2003). COPD is rare before the age of 40 and its prevalence stabilizes at 60-70 years of age (Repine, Bast, Lankhorst, & The Oxidative Stress Study Group, 1997). It is more common in men; but national studies in the USA carried out during 1979 to 1985 on patients aged 55 or more showed that because of relative changes in smoking trends in men and women, this male predominance has been fading (Feinleib et al., 1989). COPD has been named as the fourth leading cause of death in Australia in 1998 (ABS, 1998).

Ageing can be a confusing factor in the diagnosis of COPD. FEV₁/FVC ratio has negative correlation with age and height. Therefore, using this ratio to diagnose airway obstruction may over-diagnose COPD in old and tall people. To avoid over-diagnosis,

“lower limit of normal values” (LLN) which varies for each person according to age, height, gender and ethnicity have been suggested as the reference system. LLN values are statistically-derived values based on confidence intervals or the fifth percentile. Furthermore, with aging, elastic recoil properties of lung parenchyma decrease. This results in the reduction of small airway diameter as a result of dynamic compression (Bhatt & Wood, 2008); so that there is a ‘true’ airflow obstruction as part of ageing separate from any other pathological process.

Smoking is the main etiologic factor in the development of COPD. Probably, at least one-third of the global population older than 15 years is a smoker. Approximately 15-20% of smokers will acquire COPD (Halbert et al., 2003) (Hogg et al., 1994) (Fletcher & Peto, 1977) (Hogg & Timens, 2009). COPD is a leading cause of mortality and morbidity worldwide. It will probably be the third leading cause of death by 2020 (K. R. Chapman et al., 2006). The estimated death rate from COPD varies in different countries and is 4.4/100,000 in Japan and 130/100,000 in China (Chung & Adcock, 2008). Although the death rate from common fatal diseases like heart disease and stroke has been decreasing since 1970, the death rate from COPD has been increasing during this period (Jemal, Ward, Hao, & Thun, 2005). COPD is the third leading cause of disease burden in Australia (Mathers, Vos, Stevenson, & Begg, 2001). In the European Union, 56% of the health care budget for respiratory diseases is spent on COPD. Exacerbations of COPD account for the greatest burden of disease in developed countries (GOLD, 2007). This is a huge problem and healthcare issue. It is therefore amazing that relatively very little research effort has been put into it.

3-Aetiology:

As stated, *smoking* is the main cause of COPD worldwide (GOLD, 2007). Almost 90% of patients in advanced countries are either current or ex-smokers (Repine et al., 1997). In 1995, 21% of Australians aged 18 or older smoked regularly (ABS, 2001). But the development of the disease can not be explained by smoking alone. Although most COPD patients are smokers, longitudinal studies reported that only 15-20% of smokers develop COPD (Fletcher & Peto, 1977) (Jorgen Vestbo & Lange, 2002) (Hogg et al.,

1994) (Figure 2-2). Thus, in a classic 8-year longitudinal study during which subjects had lung function measured every 6 months, Fletcher et al. showed that many smokers are “resistant” to the deleterious effects of smoking and the rate of decline of FEV1 in them is quite similar to nonsmoking subjects (Fletcher & Peto, 1977). In another study in Denmark, 13.2% and 20.5% of smokers with respiratory symptoms developed COPD in 5 and 15 years respectively (Jorgen Vestbo & Lange, 2002). Therefore there must be host (presumably genetic?) factors and other environmental factors that determine the effect of smoke on the lungs, but these are poorly defined (Hogg et al., 1994; Wan & Silverman, 2009) (Hogg et al., 1994; Steven D. Shapiro & Ingenito, 2005) (Fletcher & Peto, 1977). There is a great deal of work going on trying to define COPD-related gene polymorphisms, but detailed analysis of these studies is beyond the scope of this literature review.

Genetic lack of protease inhibitor (Pi, alpha 1 anti-trypsin) is the best established cause of emphysema (Laurell & Eriksson, 1963). Apart from Pi deficiency, studies have shown that there are other predisposing genetic factors for airflow obstruction in subjects who are exposed to cigarette smoke. First-degree relatives of subjects with early onset COPD who were current or former smokers, had a greater risk of developing COPD than age matched smoking controls, despite similar pack-year smoking history (Silverman et al., 1998).

Matheson et al. in a study that recruited a random sample from the general population in Melbourne, reported that a single nucleotide polymorphism (SNP) in TNF- α 308 G→A allele was associated with COPD-related phenotypes (Matheson, Ellis, Raven, Walters, & Abramson, 2006). A SNP in the β 2-adrenergic receptor gene has also been shown to be related to human airway disease including COPD (Matheson, Ellis, Raven, Johns et al., 2006). A recently published meta-analysis and review of studies published before mid-June 2008 reviewed all papers examining genetic susceptibility to COPD (Castaldi et al., 2010). Four genetic variants were found to be significantly related to COPD susceptibility in their analysis. These four variants were GSTM1 (glutathione S-

transferase Mu1) null variant and SNPs in TGF- β 1, TNF and SOD3 (superoxide dismutase) respectively.

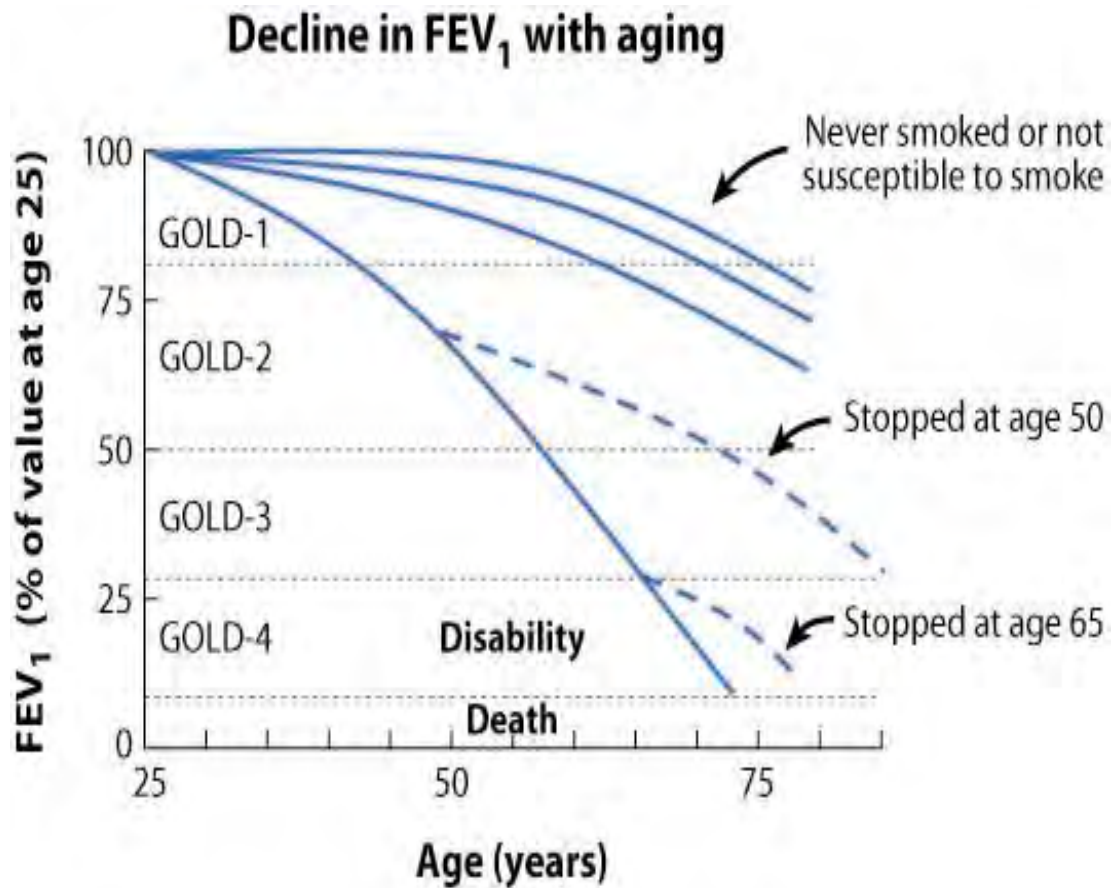


Figure 2-2. *The rate of decline of FEV1 in smokers.* Not all smokers develop accelerated rate of decline of FEV1 (Source: (Hogg & Timens, 2009)).

These emphasise the importance of oxidative stress as GSTM1 enzymes function in detoxification of environmental toxins and products of oxidative stress, and the SOD enzymes are antioxidants.

Cigarette smoking produces inflammatory infiltrations in the lungs of every smoker; this inflammation is amplified in those who develop COPD (Hogg et al., 1994) (Hogg & Timens, 2009) (Fletcher & Peto, 1977). Oxidative damage may be important in precipitating this inflammatory reaction to cigarette smoke.

There are lots of oxidants in cigarette smoke (Pryor & Stone, 1993) (Figure 2-3). Increased exposure of tissue to oxidants and decreased capacity of antioxidant mechanisms are called oxidative stress. Evidence shows links between oxidative stress and inflammation in COPD and the development of COPD (Montuschi et al., 2000; Repine et al., 1997). Reactive oxygen species, both from cigarette smoke and from inflammatory cells are toxic to cells and to tissue components such as proteins, lipids and DNA. Fibroblasts are injured by oxidants. But this injury is not limited to COPD and includes healthy smokers too (Hilbert & Mohsenin, 1996, 9-11; Morrow et al., 1995; Repine et al., 1997), and so does not fully explain COPD development.

Oxidants are counteracted by alpha1-antitrypsin action, and if the latter is deficient emphysema occurs (Larsson, 1978). Oxidants also increase mucus production by epithelial cells and impair cilia function (Repine et al., 1997). Oxidants have some direct effects including oxidation of arachidonic acid and formation of prostanoids that can increase vessel permeability and induce bronchoconstriction. Reactive oxygen species activate nuclear factor-Kappa B (NF- κ B), which is a key pro-inflammatory transcription factor and turns on many inflammatory genes (P. J. Barnes, Shapiro, & Pauwels, 2003) (Figure 2-4).

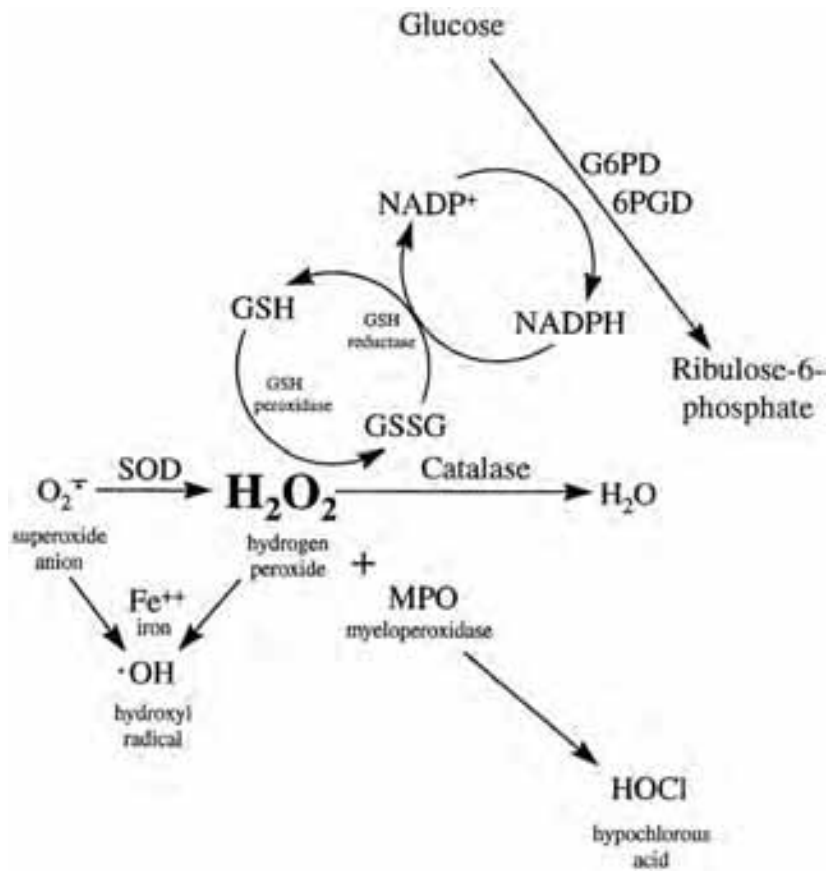


Figure 2-3. Oxygen Radical-Antioxidant Chemistry: Superoxide anion ($O_2^{\cdot-}$) formation from oxygen is the first step. $O_2^{\cdot-}$ is generated primarily in the mitochondria in the phagocytic cells. Hydrogen peroxide (H_2O_2) is the source for hydroxyl radical ($\cdot OH$) and hypochlorous acid ($HOCl$). Both $\cdot OH$ and $HOCl$ have strong oxidant activity. The main antioxidants in the lungs are superoxide dismutase (SOD), which degrades superoxide anion, and glutathione redox system that inactivates H_2O_2 . Glutathione (GSH) is another important substance. GSH plays role as a cofactor for various enzymes that reduce oxidative stress. Note that GSH is reproducible from oxidized glutathione (GSSG) by NADPH. (Source: (Repine et al., 1997))

Phosphoinositide 3-kinases (PI3K) are also activated by oxidative stress and recent reports from Peter Barnes' group in London have focused on this mechanism as being important in COPD pathogenesis. PI3K are a family of proteins that are involved in the expression and activation of inflammatory mediators, inflammatory cell recruitment, airway remodelling and resistance to corticosteroids. There are four classes of PI3Ks, IA, IB, II and III that possess lipid kinase activity; class 1 PI3Ks has been mostly investigated. Upon activation by cell surface receptors, class 1 PI3Ks phosphorylate phosphatidylinositol 4,5-bisphosphate to produce a second messenger phosphatidylinositol 3,4,5-trisphosphate. This process produces signaling molecules that are active in cell growth, apoptosis, cell movement and activation, chemotaxis and once again and importantly production of inflammatory mediators (K. Ito, Caramori, & Adcock, 2007) (P. J. Barnes, 2009; To et al., 2010).

It has been suggested that oxidative stress is also involved in glucocorticoid partial insensitivity in COPD by inactivating histone deacetylase 2 (HDAC 2), another result of PI3K activation by oxidative stress (see Section 13. Glucocorticoids) (Figure 2-5).

Noxious gases and dusts are important aetiological factors for some cases of COPD. For example, indoor air pollution due to burning biomass is an important risk factor for COPD in developing countries (R. S. Chapman, He, Blair, & Lan, 2005).

Air pollution and frequent *respiratory infections* during early life are also reported as predisposing to COPD (GOLD, 2007). In addition to above named aetiologies, low birth weight and socioeconomic status have also been emphasised amongst risk factors for COPD (Barker et al., 1991; Dharmage et al., 2009; Prescott & Vestbo, 1999; Repine et al., 1997; Thorn et al., 2007). "Childhood disadvantage" has been regarded as a significant risk factor for developing COPD. The "disadvantage factors" during early life that were predictors of accelerated FEV1 decline, and in later life lower FEV1, include maternal asthma, paternal asthma, childhood asthma, maternal smoking and childhood respiratory infection and also probably straight forward relative poverty.

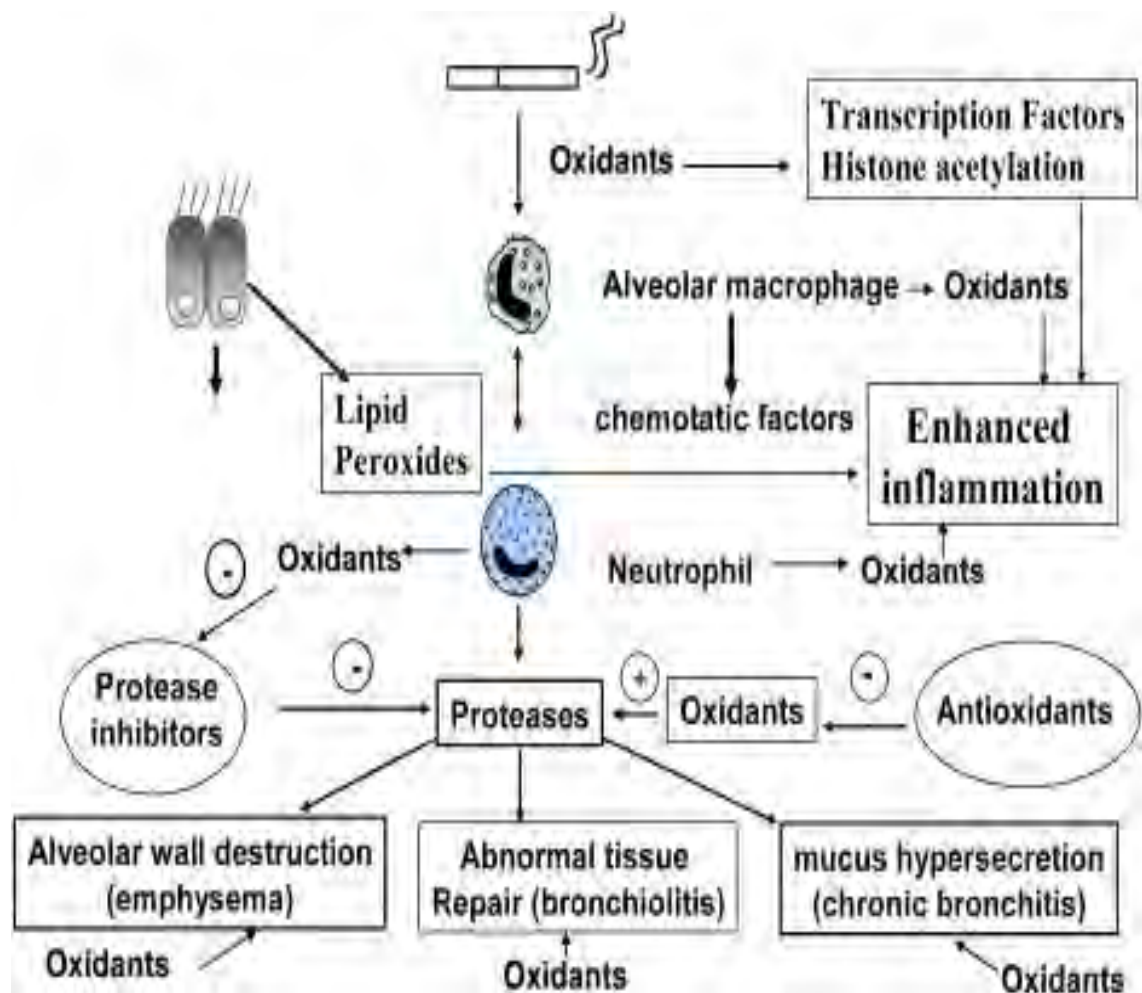


Figure 2-4. The role of oxidants in the pathogenesis of COPD. Cigarette smoke and inflammatory cells are sources of oxidants. Oxidants induce inflammation by their effects on the epithelial cells, on lipids in cell membrane, on transcription factor, histone acetylation, or decreased deacetylation. Oxidants activate proteases and inactivate antiproteases promoting alveolar wall destruction. In airways they are involved in abnormal tissue repair and in mucus hypersecretion. (Source: (Macnee, 2007))

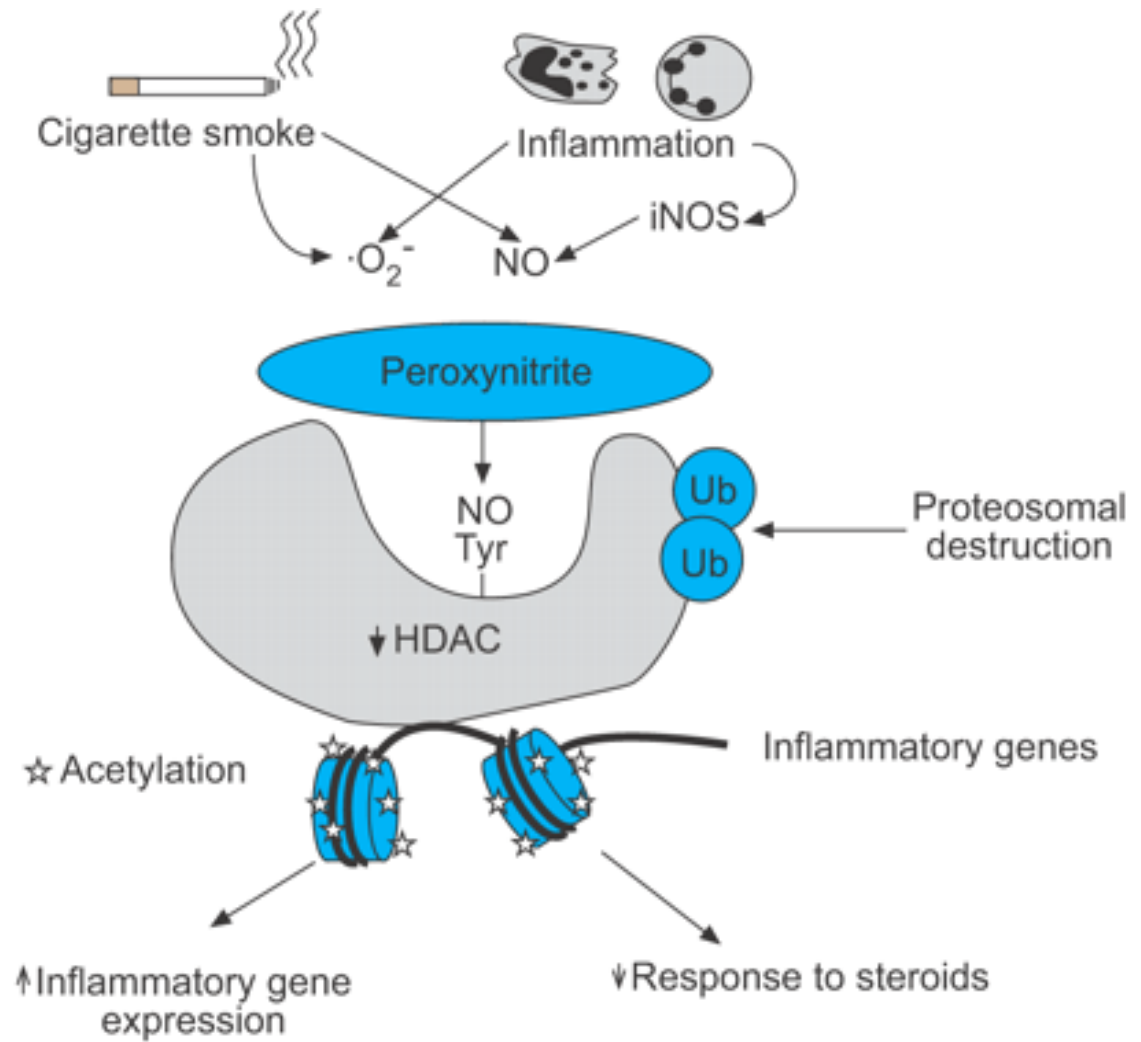


Figure 2-5. HDAC 2 reduction in smokers. Nitric oxide and superoxide anions produce peroxynitrite. HDAC 2 is inactivated and destroyed by nitration of its tyrosine (Tyr) residue by peroxynitrite. This process leads to amplification of inflammation and resistance to corticosteroids. (Source: (P. J. Barnes, Adcock, & Ito, 2005))

These factors had cumulative adverse effects on lung function and increased the risk of COPD and their impact was as large as that of heavy smoking (Bateman & Jithoo, 2006; Disano, Goulet, Muhajarine, Neudorf, & Harvey; Svanes et al.; Tabak, Spijkerman, Verschuren, & Smit, 2009).

The presence of chronic bronchitis, i.e. symptomatic cough and regular sputum production, in patients with COPD predicts an increased risk of hospitalization and amplification of FEV1 decline (J. Vestbo, Prescott, & Lange, 1996).

4-Pathology and Pathogenesis:

4.a-Introduction:

The pathological changes in COPD are the result of chronic inflammation and resulting or parallel tissue remodelling in reaction to inhalation of smoke and other types of harmful irritant gases (Hogg & Timens, 2009) (Pauwels et al., 2001). These changes happen in peripheral and central airways and the lung parenchyma (Cosio Piqueras & Cosio, 2001; Hattotuwa, Gizycki, Ansari, Jeffery, & Barnes, 2002; Hogg, 2004; Hogg, 2008) (Steven D. Shapiro & Ingenito, 2005) (Figure 2-6); although the physiological impact is thought to be in smaller airways and on lung recoil properties.

Thus, COPD is a complex disease involving all generations of airways and lung parenchyma. The literature provides more information about the pathogenesis of emphysema and small airway than large airway pathology. However, in this section I will focus on the pathology and pathogenesis in general of airway disease in COPD because it is most relevant to my project.

This section starts with a brief introduction to lung anatomy. Then emphysema, which is a very important component of COPD, is discussed very briefly because it is not centrally relevant to my work. Some airway pathological changes such as mucous gland hyperplasia have been known for decades (A. Nagai, West, Paul et al., 1985; A. Nagai, West, & Thurlbeck, 1985; L. Reid, 1954, 1960). Newer reports that discuss new

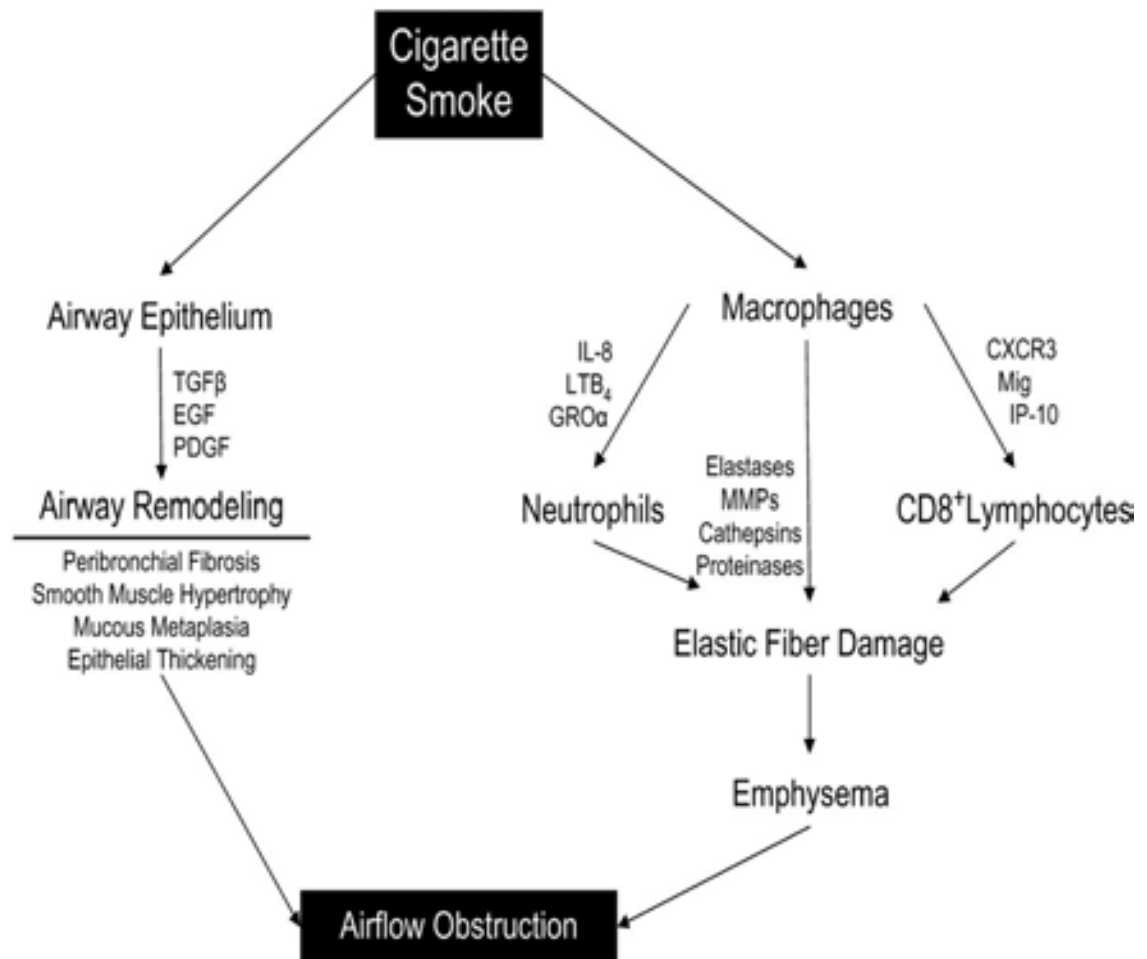


Figure 2-6. *From smoking to the development of airway obstruction.* Smoking attracts inflammatory cells to the parenchyma. Elastase from these cells causes tissue destruction. Mediators released by airway epithelium provoke airway wall thickening. (Source: (Sharafkhaneh, Hanania, & Kim, 2008)).

concepts of airway remodelling are presented in more details with emphasis on vessel and Rbm changes.

4.b-Anatomy:

The lungs have two major anatomical components: Airways and parenchyma. Airways are categorized according to their size and location. Large or central airways are close to the vocal cords, contain more and structurally organised cartilage and have a larger perimeter. Small airways are located close to the pleura, normally do not have cartilage, and their internal diameter is less than 2 mm (Lazarus, 2005). Examples of these are terminal bronchioles and respiratory bronchioles (Figure 2-7).

The lung parenchyma is the gas exchanging part of the lungs. It consists of respiratory bronchioles, atrium, alveolar ducts, alveolar sacs, and alveoli (Figure 2-8). Alveoli are completely surrounded by pulmonary capillaries. The acinus (plural form is acini) is the functional unit of the gas exchanging part of the lungs. It is the part of the lung parenchyma located distal to the terminal bronchioles (S. D. Shapiro, Snider Gordon L., Rennard Stephen I., 2005). The acinus is also called a secondary lobule (or lobule) in the literature.

In the microscopic examination of large and medium airways mucosa, submucosa, cartilage and adventitia can be distinguished (Albertine, Williams, & Hyde, 2005). Tissue obtained by fiber-optic bronchoscope (FOB) usually contains mucosa but only sometimes parts of submucosa.

Mucosa has an epithelial layer on the luminal surface (Figure 2-9). Epithelial cells are cylindric and form pseudo-stratified structures and have their own cilia. Basally, they are attached to the basement membrane (BM) which is a thin linear structure of collagen IV. BM separates the epithelium from the lamina propria (LP) which spreads between the BM and the inner border of the smooth muscle layer. The LP is made of loose connective tissue containing vessels and cells.

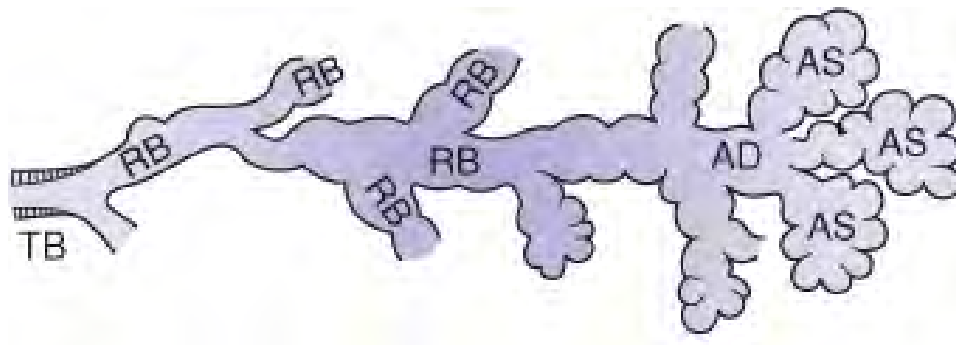


Figure 2-7. *Peripheral airways of the lungs.* Each terminal bronchiole (TB) conducts to a few respiratory bronchioles (RB). As seen respiratory bronchioles contribute to gas exchange. Alveolar ducts (AD) and alveolar sacs and alveoli follow respiratory bronchioles. Part of the lung distal to terminal bronchiole is called acinus. (Source: (S. D. Shapiro, Snider Gordon L., Rennard Stephen I., 2005)).

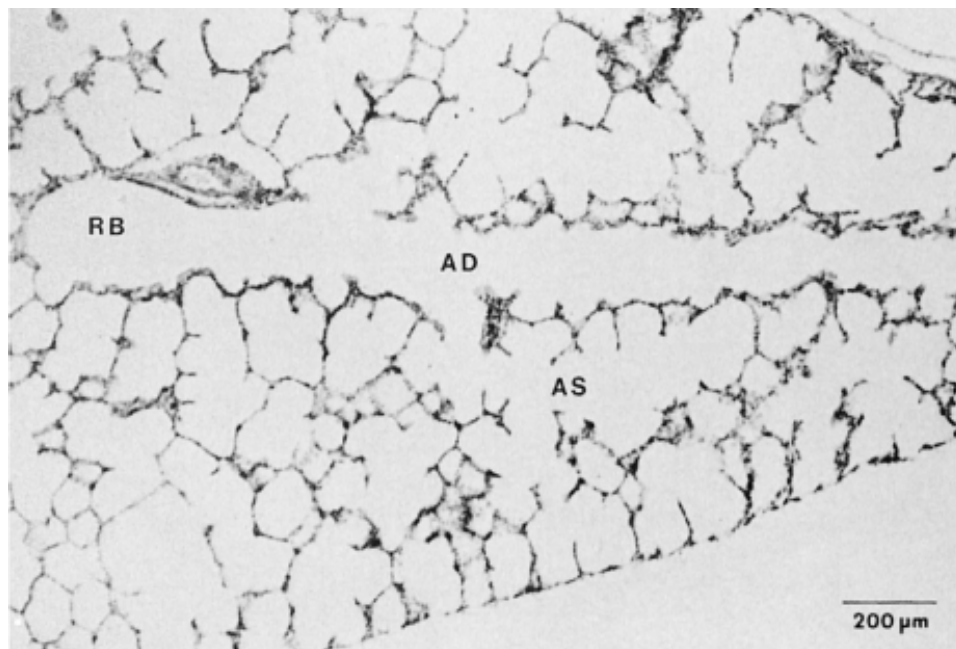


Figure 2-8. *Lung Parenchyma.* RB = Respiratory bronchiole, AD = Alveolar ducts, AS = Alveolar sacs. (Source: (S. D. Shapiro, Snider Gordon L., Rennard Stephen I., 2005)).

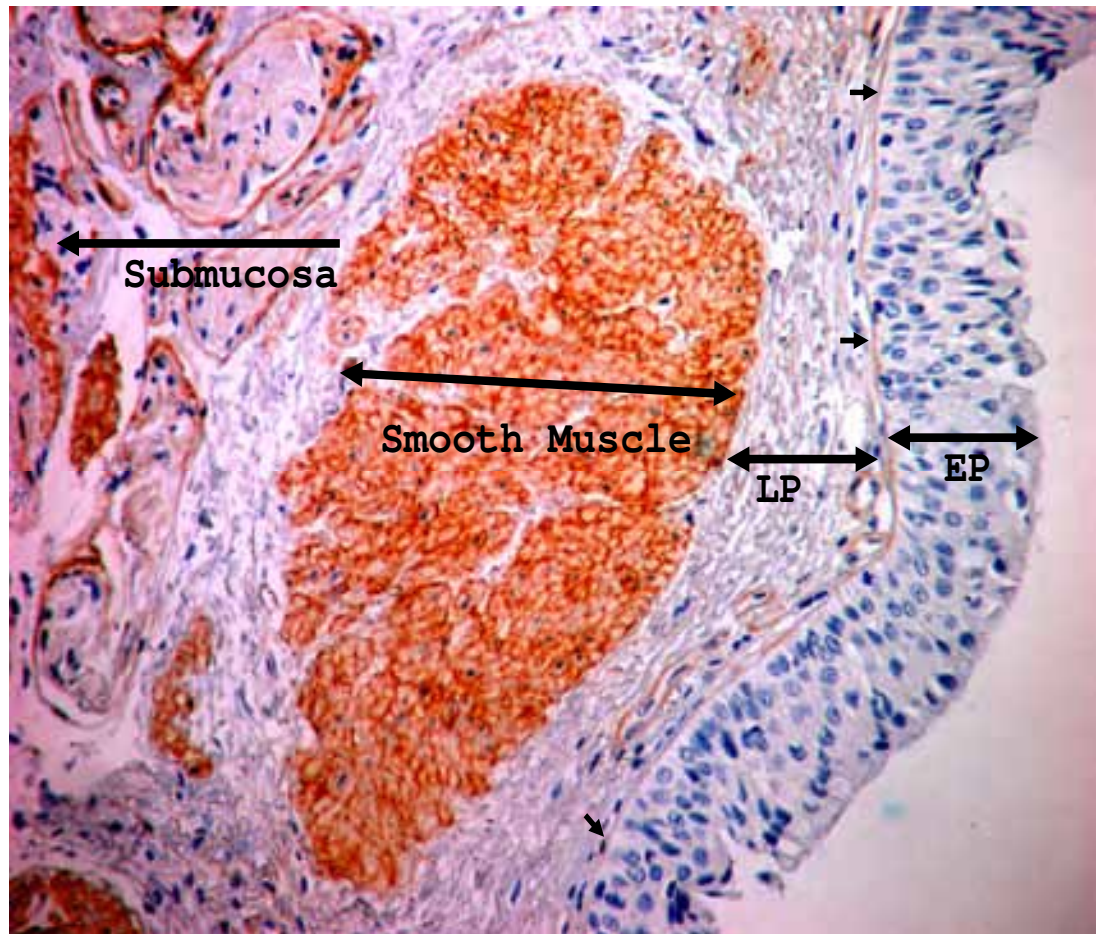


Figure 2-9. *Microscopic anatomy of airway biopsy taken by FOB.* The epithelium (EP) covers the most superficial part of mucosa and is exposed to airway lumen. It is based and attached on the basement membrane (marked by arrows) which is a linear structure. Lamina propria (LP) is made of loose connective tissue and contains blood vessels. The deepest part of mucosa is smooth muscle. Submucosa may sometimes be seen in BB and is mainly composed of mucous glands. Collagen IV antibody, x200

Smooth muscle is the deepest part of the mucosa. Deep to the smooth muscle, there is the submucosa that is mainly made of mucous glands and connective tissue (Albertine, Williams, & Hyde, 2005). Alveoli are attached to the outside of the small airway wall.

4.c-Emphysema:

Emphysema is characterized by the destruction of alveolar septae and the coalescence of alveoli to form large airspaces (Figures 2-10 and 2-11) (Liebow, 1959). Emphysema is classified by its distribution in acini (Figure 2-12). Centriacinar or centrilobular emphysema involves mainly alveoli located around respiratory bronchioles. This type of emphysema has mainly upper-lobe distribution in the lungs and is more common in smokers than panacinar emphysema. These pathological changes in cigarette smoking related COPD begin in the respiratory bronchioles. Panacinar emphysema is characterized by uniform destruction of all alveoli in the acinus, has mainly lower-lobe distribution, and is the type of emphysema characteristically seen in protease inhibitor (Pi = alpha1-antitrypsine) deficiency (Berend, 1982; Hogg & Timens, 2009; Saetta, Turato, G., Timens, W., Jeffery, P.K., 2006; S. D. Shapiro, Snider Gordon L., Rennard Stephen I., 2005).

Many mechanisms have been proposed as the primary pathogenesis of emphysema, but these mechanisms are all potentially inter-related.

Protease/ anti-protease imbalance has been the dominant mechanism proposed that could explain the evolution of emphysema since the mid 20 century (H. Kanazawa, 2007; S. D. Shapiro, Snider Gordon L., Rennard Stephen I., 2005) (Hunninghake & Crystal, 1983; H. Kanazawa, 2007; Kasahara et al., 2000; Larsson, 1978).

Matrix metalloproteinases (MMPs) may play an important role in COPD and asthma (Demedts, Brusselle, Bracke, Vermaelen, & Pauwels, 2005). Matrix metalloproteinases are a group of enzymes with collagenase, elastase, gelatinase and other types of protease

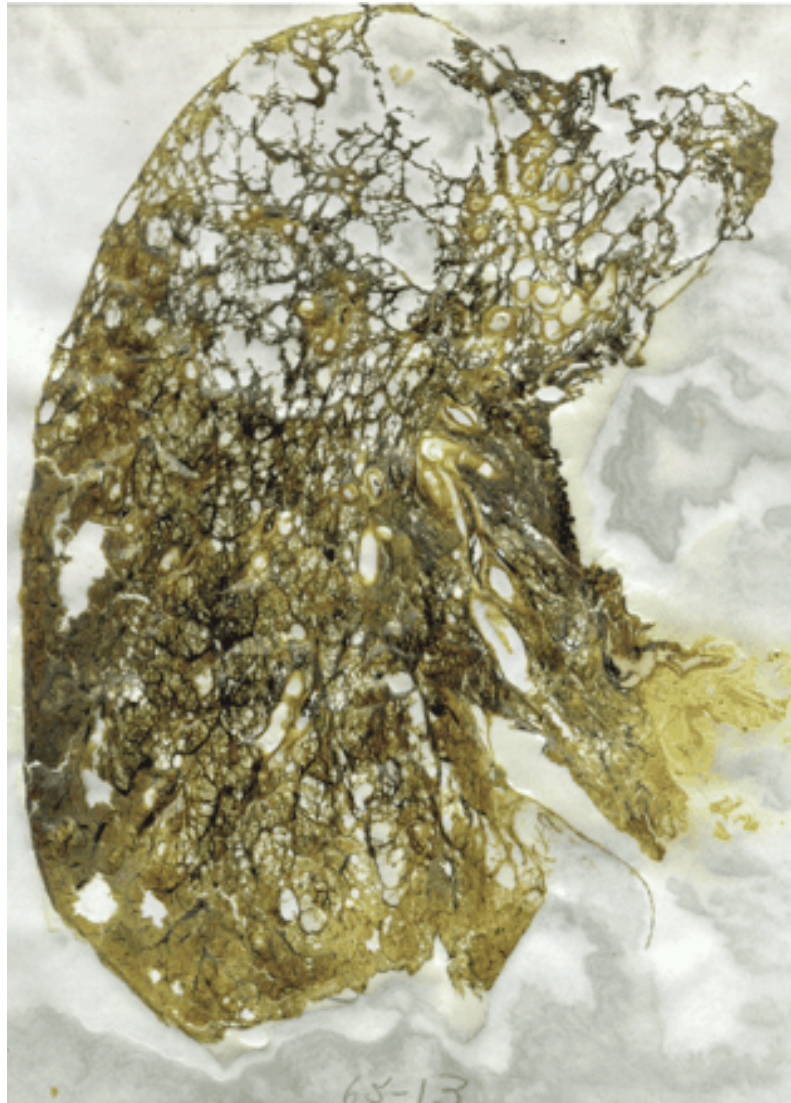


Figure 2-10. *Emphysema in thin lung section.* Note alveolar destruction in the upper lobes producing bullae. (Source: (Steven D. Shapiro & Ingenito, 2005)).

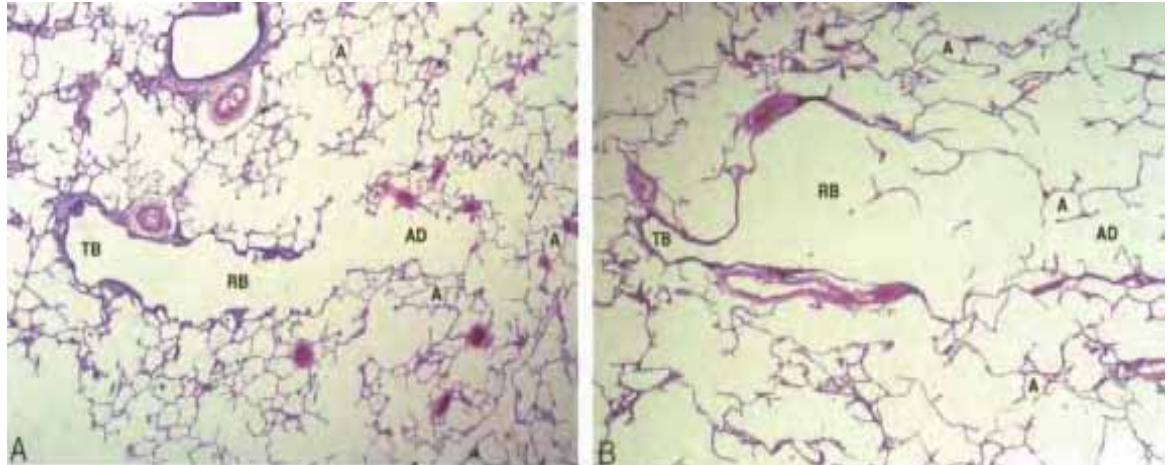


Figure 2-11. *Emphysema.* Left panel shows a section of normal lung for comparison with right panel which is from a patient with emphysema. Large airspaces in emphysema are the consequence of alveolar septae destruction and unification of adjacent alveoli. (Source: (Nishimura & Finkbeiner, 2005))

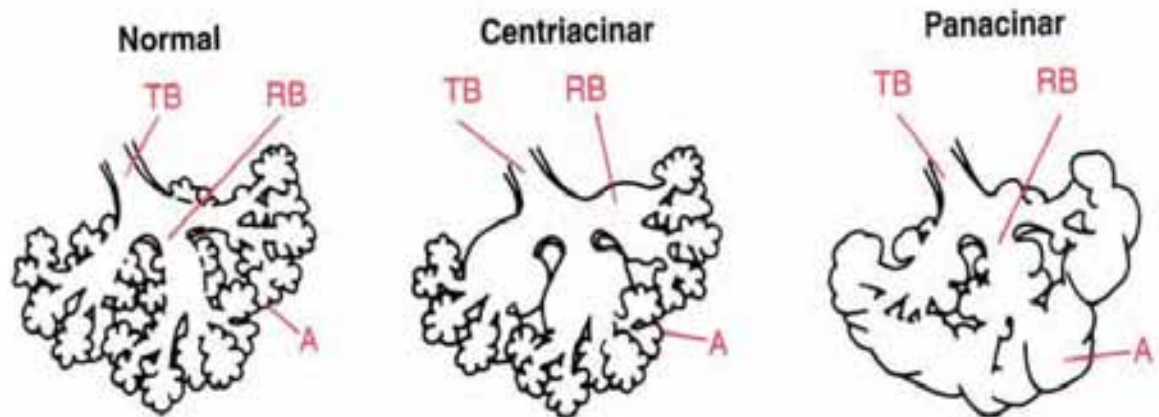


Figure 2-12. *Common types of emphysema.* In centriacinar emphysema parenchymal destruction is mainly around the respiratory bronchioles. In panacinar type destruction is seen in the whole acinus. TB = Terminal bronchiole, RB = Respiratory bronchiole, A = Alveoli. (Source (West, 2003)).

activities. Tissue inhibitors of metalloproteinase (TIMP) counteract MMP activities on tissues. Investigations have shown an imbalance of MMP/TIMP in experimental models of emphysema and also in human COPD (Lowrey, Henderson, Blakey, Corne, & Johnson, 2008; Parks & Shapiro, 2001; Prause, Bozinovski, Anderson, & Linden, 2004; R. E. K. Russell et al., 2002; Senior et al., 1991) (S. D. Shapiro, Kobayashi, & Ley, 1993) (Choe et al., 2003; D'Armiento, Dalal, Okada, Berg, & Chada, 1992; Segura-Valdez et al., 2000).

Oxidative stress due to smoking provokes *inflammation* in the lungs and airways and in the subset of smokers who are susceptible to COPD this inflammation is exaggerated (Mullen, Wright, Wiggs, Pare, & Hogg, 1985) (Retamales et al., 2001) (Di Stefano et al., 1998). Inflammation and enzyme-related proteolytic activity have been shown to have tight inter-relationships. Elastin degradation products are strong chemotactic factors and inflammatory mediators activate proteolytic enzymes (Eidelman et al., 1990; Grumelli et al., 2004; Senior, Griffin, & Mecham, 1980; Steven D. Shapiro & Ingenito, 2005; T. Zheng et al., 2000).

Vascular changes may be the pathogenetic basis of emphysema (Sharafkhaneh et al., 2008; Eugene Haydn Walters et al., 2008). Liebow in his examination of the lungs reported lack of vessels in emphysematous lungs in 1959 (Liebow, 1959).

Kasahara et al. believe that parenchymal destruction and airway obstruction in COPD is the result of apoptosis of septal endothelial and epithelial cells (Kasahara et al., 2001; Kasahara et al., 2000). Other studies support the significance of endothelial cell apoptosis in end-stage COPD (Segura-Valdez et al., 2000).

Other investigators have suggested that *autoimmune mechanisms* and *Genetic factors* may be responsible for capillary loss and evolution of emphysema (Hersh, DeMeo, & Silverman, 2008; N. Voelkel & Taraseviciene-Stewart, 2005) (Halbert et al., 2003) (Fletcher & Peto, 1977).

4.d-Airway disease:

COPD is a pan-airway disease. Association of airway involvement with clinical manifestations of disease and airway obstruction has been reported (Hogg, 2008). Small airway disease particularly contributes to the loss of function in the disease.

4.e-Small airways:

Small airways are surrounded by lung parenchyma and show marked pathological changes in COPD. Small airways do not normally contain cartilage, therefore their wall is not as stiff as large airways and their luminal diameter is dependent on pleural pressure (Lazarus, 2005). Emphysema reduces parenchymal attachments and recoil support for small airways. Therefore, during expiration when pleural pressure increases, they are easily collapsible. Small airway involvement has a major impact on lung function and is very important in the physiopathology of COPD. An animal study has shown both fibrosis and thickening of small airway walls and emphysema after exposure to smoke (Wright & Churg, 1990), these inducing two impacts on the small airway lumen (direct narrowing and dynamic compression secondary to emphysema and loss of elastic recoil). Small airway involvement due to smoking begins years ahead of clinical COPD (Niewoehner, Kleinerman, & Rice, 1974). In this post-mortem study, young male smokers had respiratory bronchiolitis and accumulation of pigmented alveolar macrophages. Few nonsmokers showed the same changes (Niewoehner et al., 1974). A Canadian study found the same small airway changes and showed the severity of pathological findings was associated with deterioration of lung function (Cosio et al., 1978).

In severe emphysema, the small airways have thicker walls but a looser collagen structure. This happens because the decorin content of extracellular matrix (ECM) is reduced; normally decorin cross links between collagen fibrils and provides strength to the ECM (van Straaten et al., 1999) (Postma & Timens, 2006).

4.f-Large airways:

Information about pathologic characteristics in large airways in COPD is limited to classic descriptions of epithelial goblet cell hyperplasia and squamous metaplasia,

submucosal mucous gland hyperplasia (Figure 2-13 & 2-14), mucus hypersecretion and inflammatory cell infiltration of the lamina propria and submucosal glands (L. Reid, 1954, 1960; Eugene Haydn Walters et al., 2008) (Jeffery, 1991; Mullen et al., 1985; O'Shaughnessy, Ansari, Barnes, & Jeffery, 1997; Steven D. Shapiro & Ingenito, 2005).

Professor Lynne Reid reported mucous gland hyperplasia as a landmark pathological finding in the airways of patients with chronic bronchitis (L. Reid, 1954, 1960) (Figure 2-13). Gland/wall ratio (thickness of the airway wall from epithelial BM to the margin of cartilage divided by the thickness of mucous gland layer) was much higher for patients with chronic bronchitis than normal controls; and this was suggested as a cause of airflow obstruction.

4.g-Airway remodelling:

Airway remodelling is defined as structural changes in the airway wall that appear during the course of chronic airway disease (Boulet & Sterk, 2007) (Elias et al., 1999). Both inflammatory and resident cells are thought to contribute to airway remodelling in chronic inflammatory airway diseases (Postma & Timens, 2006). As previously said, there are only limited reports about remodelling in large airways using BB from COPD patients (E. Haydn Walters, Johns, & Ward, 2010). In contrast, remodelling in asthma has been under close examination during the last 2 decades. Changes in all layers of the airway wall have been reported in asthma (Elias et al., 1999; E. Haydn Walters et al., 2010). Epithelial shedding (Naylor, 1962) (Rennard, 1996), Rbm thickening (Dunnill, 1960) (Bousquet et al., 2000; Liesker et al., 2009) (Bergeron & Boulet, 2006; Elias et al., 1999; Postma & Timens, 2006; Rennard, 1996; Roche et al., 1989) (Cutz, Levison, & Cooper, 1978; Jeffery et al., 1992) vascular changes (discussed later in details), smooth muscle hypertrophy and hyperplasia (Carroll et al., 1993) (Dunnill et al., 1969) (Bousquet et al., 2000) mucous gland hypertrophy (Dunnill et al., 1969) (Carroll et al., 1993), increase of epithelial goblet cells (Aikawa, Shimura, Sasaki, Ebina, & Takishima, 1992) and even degeneration of cartilage (chondrocytes) have been reported in asthma (Haraguchi, Shimura, & Shirato, 1999) (Figure 2-15). Tissue remodelling in COPD is complex and, as discussed above, consists of both

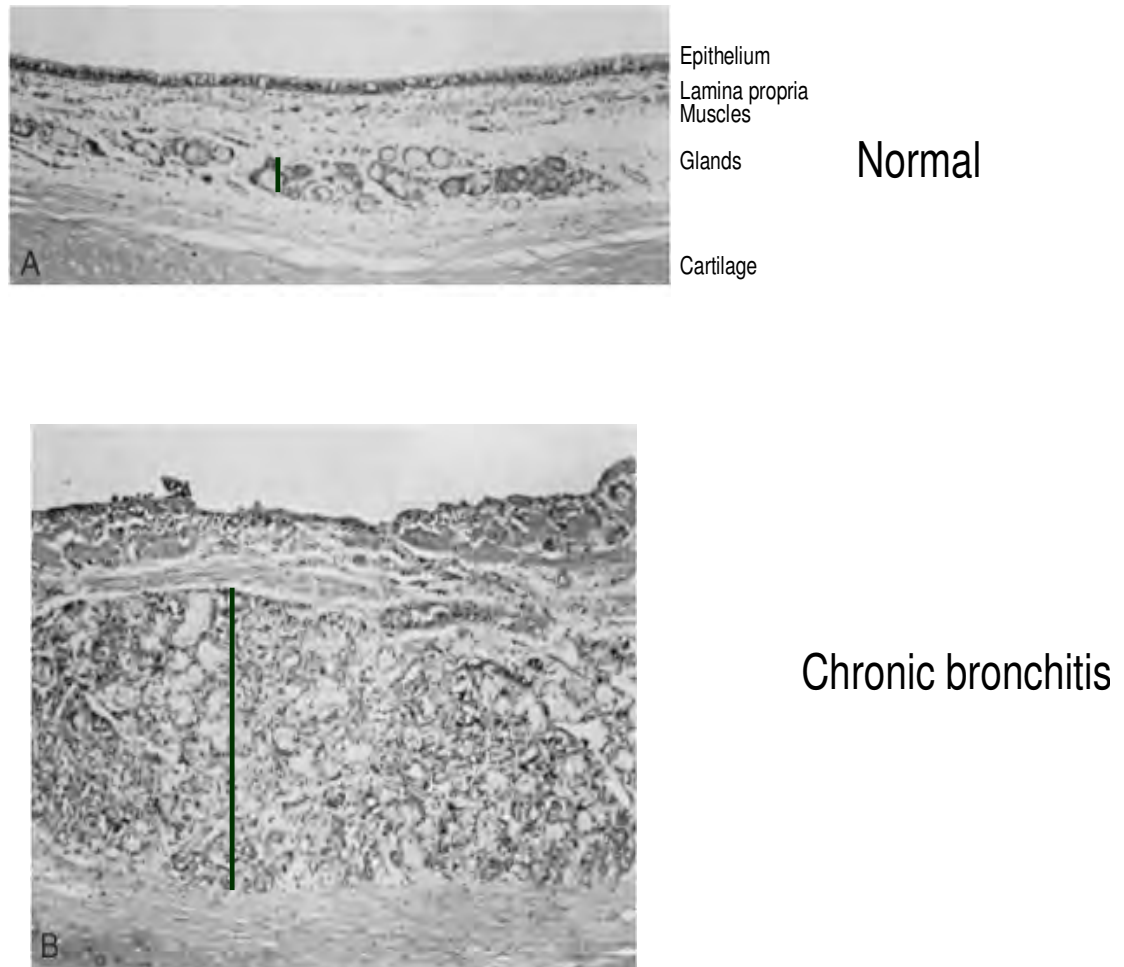


Figure 2-13. *Mucous gland hyperplasia.* The thickness of the mucous gland layer (marked with straight line) is compared in normal and COPD subjects. (Source: (S. D. Shapiro, Snider Gordon L., Rennard Stephen I., 2005)).

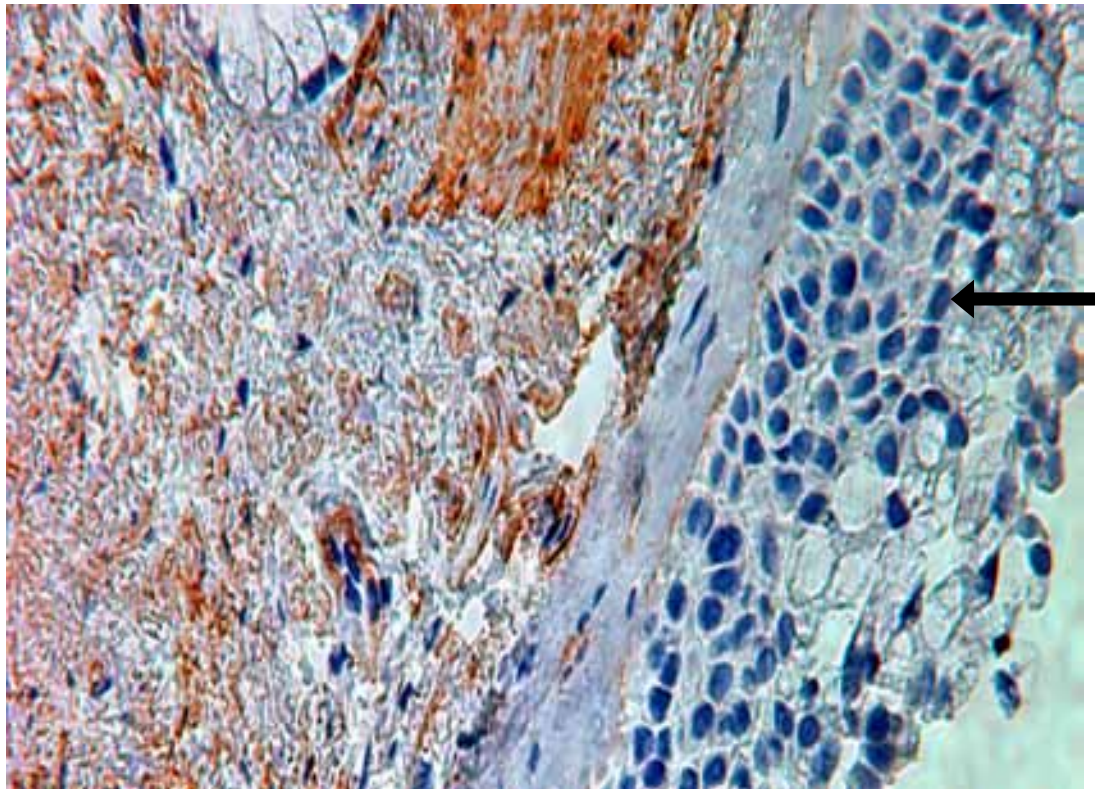


Figure 2-14. *Squamous metaplasia in COPD (arrow).*

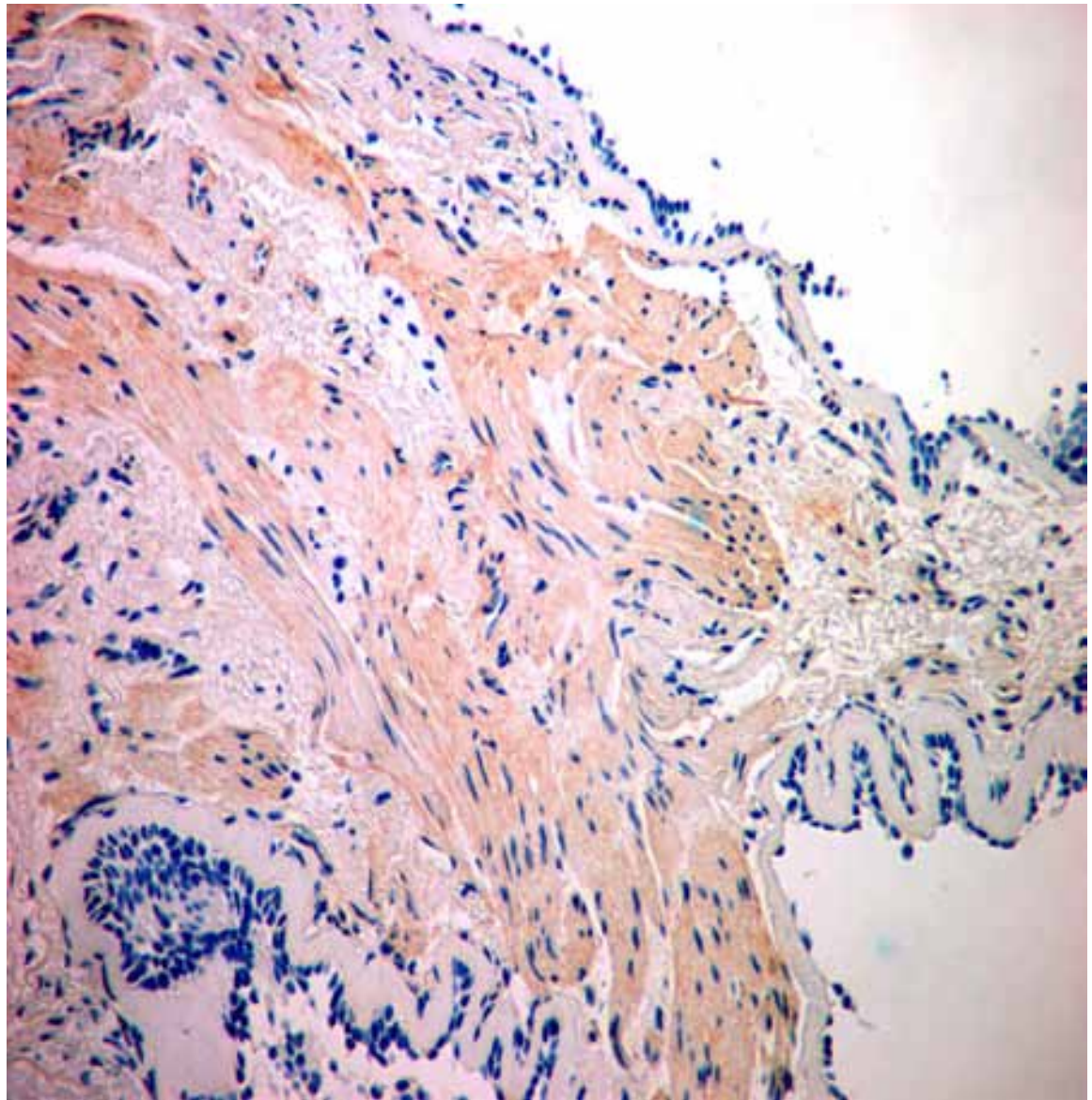


Figure 2-15. *Airway remodelling in asthma.* This biopsy belongs to a patient who suffers from asthma. Significant structural changes are marked in this slide. You can see that there is epithelial shedding, some parts of basement membrane are naked, basement membrane is thickened, and muscle has occupied most parts of mucosa. Collagen IV- X
200

destruction in the parenchyma and structural changes in the airway walls (Zandvoort et al., 2006).

The *basement membrane (BM)* of epithelium has two components: true BM or basal lamina and reticular BM (Rbm) or lamina reticularis. The true BM which has two layers, lamina rara and lamina densa, separates the epithelium from the mesenchyme and mainly is made of Collagen IV, laminin and proteoglycans. Collagen IV is a fibrous protein that provides strength, rigidity and resilience to tissue. Laminin is an adhesive protein. Proteoglycans are structural proteins that can entrap water molecules to form a suitable ground material for insoluble fibrous proteins to stay in extracellular matrix. Collagen I, III, V, fibronectin and tenascin are the major components of the *reticular basement membrane (Rbm)*. The Rbm is located immediately beneath the true BM. In contrast to what the earlier reports (Dunnill, 1960) suggested, it is now clear that it is actually the Rbm that thickens in asthma and not the true BM itself. These structures form a loose mesh beneath the true BM in normal subjects (Liesker et al., 2009) (Bousquet et al., 2000) (Roche et al., 1989) (Bergeron & Boulet, 2006) (Postma & Timens, 2006) (Rennard, 1996). Rbm thickening is probably an early event in asthma (Cutz et al., 1978). Electron microscopic studies in asthma have confirmed that the true BM (lamina rara and lamina densa) has normal thickness and appearance (Roche et al., 1989).

The few reports about Rbm thickness in COPD have been contradictory. A recently published paper comparing COPD subjects with physiologically normal ex-smoking controls reported increased thickness of Rbm in COPD (Liesker et al., 2009). The control group had similar pack-year history of smoking as the COPD group. Three COPD subjects were never smokers and the rest were ex-smokers. However, other papers have reported no significant difference between normal and COPD subjects (Jeffery, 2004) (Chanez et al., 1997). Chanez et al. categorized COPD subjects as responders and nonresponders according to the reversibility of airflow obstruction to oral glucocorticoids. They did not find a significant difference between non-responders and control groups for Rbm thickness. However, corticosteroid responders who also had

higher absolute number of eosinophils and eosinophil cationic protein in their BALF, had a trend towards thicker Rbm than the control group ($p = 0.05$) and significantly thicker Rbm than non-responders, suggesting some overlap with asthma pathology.

Another group, in an abstract published in 1996, compared the Rbm thickness in asthma, chronic bronchitis with normal lung function, chronic bronchitis with airway obstruction and normal groups. They found no significant difference between subjects with chronic bronchitis without airway obstruction, subjects with chronic bronchitis with airway obstruction and the normal control group (Jeffery, 2001), all in contrast to asthma.

Smooth muscle thickness in asthmatics increases as a result of hypertrophy and hyperplasia (Jeffery, 2001). Dunnill, a pathologist in Oxford, did not find significant change in smooth muscle thickness in COPD subjects who died of their disease in his classic work comparing airways in asthma, COPD and controls (Dunnill et al., 1969).

Changes to the *ECM* happen in airway remodelling (Postma & Timens, 2006). Aberrant deposition of collagen results in small airway wall thickening in COPD (Hogg et al., 2004; Hogg & Timens, 2009). It is not only the quantity, but also the quality of ECM that may change. As mentioned already, decorin is a component of ECM that provides strength to the tissue by cross-linking between collagen fibrils. In severe emphysema decorin is reduced. Therefore small airways have thicker airway walls that are paradoxically weaker and more collapsible (Postma & Timens, 2006).

Mucus gland hyperplasia has been reported in both asthma and COPD (Dunnill et al., 1969) (L. Reid, 1960).

Degeneration of *cartilage* (chondrocytes) and perichondrial fibrosis have been reported in both chronic bronchitis and emphysema in a postmortem study (Haraguchi et al., 1999); and certainly clinically chondromalacia causing intense dynamic airway compression in expiration is well recognised (Inoue, Hasegawa, Nakano, Yamaguchi, & Kuribayashi, 2009).

4.h-Airway vascularity and angiogenesis:

Angiogenesis means vascular development and new vessel formation. Angiogenesis has attracted much attention recently in the study of lung diseases. Lung vessel homeostasis maintains specific density of vessels per unit area of the lungs. Injury can interfere with this homeostasis and result in chronic lung disease (N. F. Voelkel, Douglas, & Nicolls, 2007). Voelkel et al. in their review paper suggested that in COPD this maintenance mechanism probably fails (N. Voelkel & Taraseviciene-Stewart, 2005), but direct evidence for this is fragmentary at least.

Vessels probably play a key role in airway remodelling. Angiogenesis is a component of remodelling in chronic inflammatory diseases including airway diseases (Dunnill, 1960; Bryce N. Feltis et al., 2006; Li & Wilson, 1997; Makinde, Murphy, & Agrawal Devendra, 2006; Eugene Haydn Walters et al., 2008) (Knox, Stocks, & Sutcliffe, 2005). Hypervascularity of airways in asthma is well recognised (Li & Wilson, 1997) (Orsida et al., 1999; Salvato, 2001). Its correlation to asthma severity and bronchial hyper-responsiveness (BHR) and bronchodilator reversibility (BDR) has been reported (Hashimoto et al., 2005; Orsida et al., 1999; Salvato, 2001; Vrugt et al., 2000).

Hashimoto et al., using CD31 monoclonal antibody to mark the vessels, compared vascularity of small and medium sized airways of subjects with moderate COPD and subjects with asthma to normal controls. All specimens were taken during a thoracotomy for peripheral lung carcinoma presenting as a nodule. Control subjects were nonsmokers and all COPD subjects had a history of smoking. They reported greater vascular area between the epithelial basement membrane and outer border of smooth muscle in COPD subjects compared to controls (Hashimoto et al., 2005). They also compared the specimens for VEGF immuno-staining and did not find any difference between the COPD and control groups in VEGF positive cells. Typically, this study like other studies that use peripheral lung biopsies taken during thoracotomy for the study of airways, could not recruit normal control volunteers. Because the investigation did not include a group of smokers with normal lung function, they were not able to attribute their

findings to COPD because their findings could have been caused by smoking per se. There may also be confounding by changes more specific to presence of neoplasia.

Kuwano et al. using trichrome to stain vessels compared the peripheral airways in asthmatics, normal controls and patients with mild COPD. The number of vessels per square mm of tissue (vessel density) was not statistically different in the groups (Kuwano et al., 1993).

A group in Italy investigated airway remodelling in BB taken from central airways in smokers with normal lung function and in those with COPD (Calabrese et al., 2006). They recruited 8 healthy non-smokers, 9 smokers with GOLD 0 classification (normal lung function) and 9 smokers with GOLD 2 classification (moderate COPD). None of the smokers showed CT manifestations of emphysema (in reality air-trapping). The subjects were not using corticosteroids. The investigators used monoclonal antibody against Collagen IV to mark the vessels, anti-VEGF antibody and anti-integrin $\alpha\beta 3$ antibody to detect VEGF-positive cells and integrin $\alpha\beta 3$ positive vessels in the lamina propria [Integrin $\alpha\beta 3$ (alpha nu beta) is an adhesion molecule that is not expressed on the resting endothelium. It is upregulated in new capillaries proliferating with angiogenic stimuli]. They found more vessel numbers per unit area of the lamina propria and higher vascular area (defined as vascularity% or the area of vessels divided by the area of the lamina propria) in GOLD 0 (chronic bronchitis without airway obstruction) and GOLD 2 participants compared to normal non-smokers. GOLD 0 subjects also had significantly more vessels, but not more vascular area, than GOLD 2. They also found more VEGF positive cells in the lamina propria of GOLD 0 and GOLD 2 subjects compared to normal nonsmokers. GOLD 2 subjects had a higher number of VEGF positive cells than GOLD 0. Both the number of vessels and area of vessels positive for integrin $\alpha\beta 3$ were higher in GOLD 0 and GOLD 2 than non-smokers. This study could not find any relationship between histological and clinical/functional data.

The investigators concluded that an angiogenic process is active in smokers with or without airway obstruction; but this angiogenesis is not related to air flow limitation.

The authors added that their study was the first one that had examined integrin $\alpha v \beta 3$ in bronchial tissue of COPD patients. They suggested that integrin $\alpha v \beta 3$ interacts with VEGF to exert angiogenic effects. VEGF and integrin $\alpha v \beta 3$ may work together to regulate vessel formation.

Recently Zanini et al. found higher vascular area in the LP of BB from 10 COPD subjects compared with 8 controls who had presented with haemoptysis or a peripheral pulmonary nodule. COPD subjects were not on ICS. All COPD subjects had quit for 10 years or more and suffered from moderate to severe disease. The control group consisted of life-long nonsmokers. However, vessel numbers were not different (Zanini et al., 2009), suggesting the change was only of larger vessel size. Zanini et al. also found a higher number of VEGF positive cells in the COPD group. The control subjects cannot be considered as normal controls because they had clinical and/or radiographic manifestations of disease. The COPD patients consisted only of long-term quitters; which is not very typical or representative of COPD patients in the community, nor in daily clinical practice.

4.i-Angiogenic factors:

There are numerous angiogenic factors including members of the fibroblast growth factor (FGF) family, VEGF, angiogenin, TGF- α and TGF- β , platelet-derived growth factor (PDGF), tumor necrosis factor alpha (TNF- α), hepatocyte growth factor (HGF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukins, chemokines, and angiopoietin 1 and 2.

VEGF is the most potent vascular growth factor discovered and its level is often increased in inflammation (Puxeddu, Ribatti, Crivellato, & Levi-Schaffer, 2005) (Jackson, Seed, Kircher, Willoughby, & Winkler, 1997) (Levi-Schaffer & Pe'Er, 2001) (Knox et al., 2005). There are six proteins in VEGF family; from A to F. In human tissues VEGF₁₆₅ is the predominant isoform followed by VEGF₁₂₁ and VEGF₁₈₉

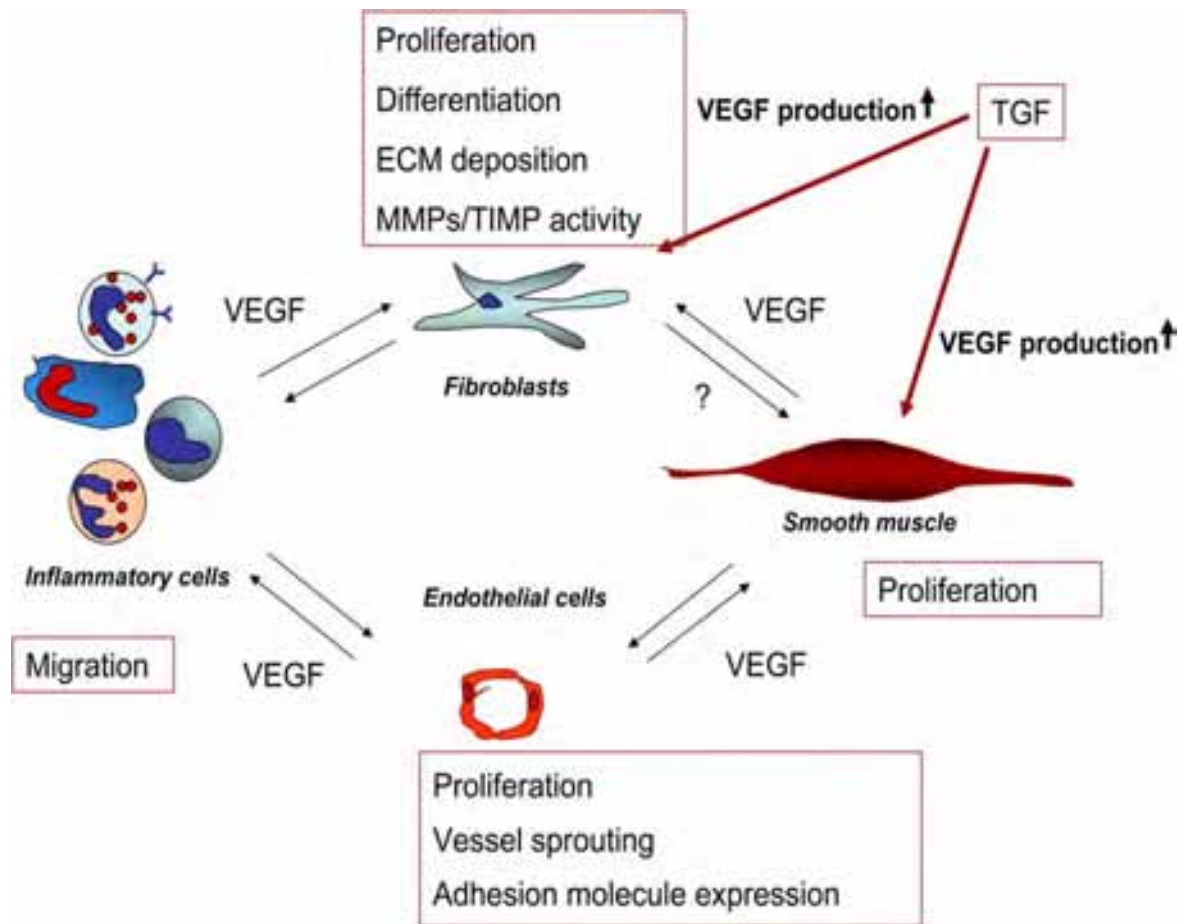


Figure 2-16. Schematic representation of the main regulatory roles of vascular endothelial growth factor (VEGF). ECM = extracellular matrix; TGF = transforming growth factor; MMP = matrix metalloproteinase; TIMP = tissue inhibitor of metalloproteinase. (Source:(Postma & Timens, 2006))

(Berkman et al., 1993). VEGF is a potent multifunctional cytokine with multiple effects on endothelium (Figure 2-16).

VEGF induces its function through specific receptors. These receptors belong to tyrosine kinase family and are named VEGFR-1 (Flt-1, fms-like tyrosine kinase), VEGFR-2 (KDR/FLK-1, Kinase-insert domain receptor/fetal liver kinase) and VEGFR-3 (flt4, fms-like tyrosine kinase 4). VEGFR-1 works as a decoy receptor and therefore has inhibitory function. VEGF has its angiogenic activity through VEGFR-2. These KDR receptors are specifically found on endothelial cells (Kranenburg et al., 2005; Tammela, Enholm, Alitalo, & Paavonen, 2005) (Thomas, 1996). VEGF transfers its signals to the endothelial cells by mainly using VEGFR-2. VEGFR-3 is seen on all endothelial tissues during development but, in adulthood its presence is limited to lymphatic endothelial cells. Proliferation, sprouting, migration and tube formation of endothelial cells happen in the presence of VEGF.

Importantly VEGF expression is higher in areas where vessel formation is happening (Ferrara, Gerber, & LeCouter, 2003; Tammela et al., 2005). Anti-VEGF antibody blocks the vascular growth factor activity of VEGF (Simcock et al., 2007) and was first used clinically over 10 years ago (Mordenti et al., 1999; Shaheen et al., 1999) and has become an established treatment in ophthalmology and oncology {Tol, 2010 #461}. VEGF is necessary for survival of endothelial cells. It induces the expression of anti-apoptotic factors in the endothelial cells (Tammela et al., 2005). Mice lacking VEGF die during intra-uterine life (Risau, 1997). In vitro studies showed that VEGFR-2, not VEGFR-1 receptors induce the effects of VEGF (Gerber, Dixit, & Ferrara, 1998).

In inflammatory diseases, increasing need for oxygen and the presence of inflammatory mediators stimulate VEGF production. This has been shown in chronic inflammatory diseases like rheumatoid arthritis and psoriasis (Jackson et al., 1997; Nagashima, Yoshino, Ishiwata, & Asano, 1995).

Cigarette smoke extract reduced VEGF protein in animal and human studies (K. Nagai, Betsuyaku, Ito, Nasuhara, & Nishimura, 2005; Tudor, Wood, Taraseviciene, Flores, & Voekel, 2000). In contrast, Hiroshima et al. found higher number of vessels in areas with squamous metaplasia in smokers compared to normal mucosa in nonsmokers (Hiroshima et al., 2002).

Studies have suggested higher VEGF and its receptor levels in asthma airways and positive relationship between VEGF, and in some studies other angiogenic factors, and vascularity in asthma (Bryce N. Feltis et al., 2006; Hoshino, Nakamura, & Hamid, 2001; Hoshino, Takahashi, & Aoike, 2001). Another study found higher VEGF/endostatin ratio in asthma that responded to ICS (Asai et al., 2002). Both VEGF and angiogenin showed significantly positive correlation with severity of asthma and negative correlation with FEV1 percent predicted (Abdel-Rahman AM, 2006).

Angiogenin is a single-chain polypeptide that belongs to the ribonuclease superfamily with 33% sequence homology to the pancreatic ribonuclease A. Its ribonuclease activity is weak but, is necessary for its angiogenetic activity. It is a potent angiogenic factor and was first derived from tumor cells in culture media (Abdel-Rahman AM, 2006; Gao & Xu, 2008; Klagsbrun & D'Amore, 1991) (Strydom, 1998; Tello-Montoliu, Patel, & LIP., 2006). Mast cells have been shown to store and secrete angiogenin in response to a variety of stimuli (Kulka, Fukuishi, & Metcalfe, 2009).

Angiogenin induces new vessel formation by stimulating endothelial cell migration, proliferation and formation of vessel tubules. To stimulate endothelial cell proliferation, angiogenin binds to endothelial cell surface receptors that are different from actin binding proteins. However, by binding to the cell surface actin, angiogenin can also activate proteolytic enzymes (collagenases) which degrade laminin and fibronectin of the endothelial BM and therefore facilitates endothelial cell migration towards the angiogenic stimulus, i.e. angiogenin in the perivascular tissue (Gao & Xu, 2008; Strydom, 1998; Tello-Montoliu et al., 2006). Probably angiogenin plays a comprehensive role in vessel formation from migration of endothelial cells towards the

stimulus for vessel formation following tissue damage which is facilitated by disintegration of the BM after binding of angiogenin with cell surface actin, endothelial cell proliferation and finally maturation of newly formed capillary walls by migration and proliferation of smooth muscle (Tello-Montoliu et al., 2006).

A study examined resected lung tissue from six patients with severe emphysema who were current smokers with eleven nonsmoking or remote ex-smoker control subjects (Kasahara et al., 2001). They found VEGF, VEGFR-2 and mRNA expression was reduced in emphysema.

One study has found higher than normal concentration of VEGF in central and peripheral airways and lung parenchyma in COPD, with VEGF having an inverse correlation with FEV1. VEGFR receptors were also increased in COPD subjects in this study (Kranenburg et al., 2005). This was a very complex study and it is not a paper easy to interpret and its findings were contrary to the Kasahara et al. study (see above) that showed increased alveolar endothelial and epithelial cells death due to a reduction of VEGF in emphysema (Kasahara et al., 2001) (Kasahara et al., 2000) and Koyama et al. study that reported VEGF reduction in BAL fluid of smokers (Koyama et al., 2002). Study subjects included 14 control subjects with normal lung function and 14 COPD patients who underwent thoracotomy and lung resection for lung cancer. Both peripheral and central airway tissues were used for the study. All subjects had a history of smoking but were a mixture of current- or ex-smokers. Pack-year history and age were not significantly different between the two groups. No one had been on glucocorticosteroids within 3 months before surgery. Two blinded examiners used a semiquantitative method for assessing VEGF and its receptor levels. Inter-observer reproducibility was evaluated by Pearson's correlation test.

VEGF and its receptors were assessed in the bronchial epithelium, mucosal and submucosal vessels, airway smooth muscle cells and macrophages in bronchi. In periphery, bronchiolar and alveolar epithelium, bronchiolar smooth muscle cells and macrophages in bronchioles and alveoli were examined. The investigators correlated

their findings with TGF- β 1 expression obtained from a previous study, which was performed on the same subjects. VEGF and its receptors were detectable in the epithelium, smooth muscle cells, bronchial microvasculature of the mucosa and submucosa, and inflammatory cells (predominantly macrophages) of the bronchi. In the vessel wall VEGF and receptors were detectable in vascular smooth muscle cells, while endothelial cells were positive only for VEGF receptors and not VEGF itself (Kranenburg et al., 2005).

In the COPD group compared to the control group, bronchial VEGF staining was significantly higher in airway smooth muscle cells, but not in the epithelium nor in macrophages. VEGF expression was also higher in vascular smooth muscle cells in the mucosal and submucosal vessels of the large airways. The levels of both KDR/Flk1 (VEGFR-2, active receptor) and Flt-1 (VEGFR-1, decoy receptor) were not significantly different between the groups (Kranenburg et al., 2005).

In the peripheral region, VEGF and VEGFR-1, but not VEGFR-2, staining was significantly intensified in the bronchiolar epithelium of COPD patients. VEGF, but not its receptors, was slightly increased in airway smooth muscle cells. VEGF and VEGFR-2 expression was higher in vessel smooth muscle cells in the COPD group. Again endothelial cells did not contain VEGF but, the expression of endothelial VEGFR-2 and 1 were increased in the COPD group. Macrophages in the peripheral airways, but not in the alveolar region, of COPD patients showed higher VEGF. Neither VEGF receptor showed any differences between the bronchioles and alveoli of the two groups. There was an inverse correlation between FEV1 and VEGF content of bronchial mucosal vessels and smooth muscle cells of airways, but only if the two groups were analysed together which does not mean very much given the between group analyses above. The study found a significant positive correlation between VEGF and TGF- β staining in the epithelium (Kranenburg et al., 2005).

The authors concluded that COPD is associated with higher than normal concentration of VEGF in central and peripheral airways and also lung parenchyma; and that VEGF

has an inverse correlation with FEV1 but this was a technically anomalous analysis (Kranenburg et al., 2005). VEGFR receptors were also increased in COPD subjects. They believed that their findings show that VEGF and its receptors are involved in airway remodelling in COPD. The authors suggested that the relationship between VEGF and TGF- β 1 showed the link between inflammatory mediators and tissue remodelling. Kranenburg et al. commented that their findings suggest an attempt by the lungs to repair the damage from smoking and that VEGF and its receptors participate in the repair process. They also stated that their results were different from the results of Kasahara et al. (who found lower VEGF in lung parenchyma) because the subjects recruited by Kasahara et al. were purely emphysematous (Kasahara et al., 2001) and their subjects were mild to moderate COPD and were not classified as having chronic bronchitis or emphysema. Moreover, the subjects with emphysema at Kasahara et al.'s study had severe airway obstruction.

There are some notable points to be made about the Kranenburg et al.'s rather complex report:

First, although it is clear that all subjects had a history of smoking, we do not know what proportion of each group were current- or ex-smokers. Therefore, it is not possible to discriminate between the effects of smoking from the effects of having COPD itself, as defined by the GOLD classification. Furthermore, the COPD group was compared with a control group that consisted of subjects with a history of smoking who were under investigation or treatment for peripheral lung cancer; therefore they were not technically normal controls, and indeed it is not really obvious what they represent as a rather "mixed bunch".

Second, inter-observer reproducibility of measurements was tested by Pearson's correlation, which may not be a reliable way to test reproducibility (J.M. Bland & Altman, 1986). Indeed, the statistical analysis is rather suspect, and their correlation analyses quite inappropriate and indeed meaningless.

Kanazawa et al. compared VEGF level in sputum of COPD patients and asthma patients with VEGF level in sputum of normal individuals. They divided COPD patients to those who had emphysema (diagnosed by HRCT) and those who did not. All COPD subjects had a significant smoking history. None of the normal controls or asthmatics was a smoker. Asthma subjects were not on ICS. They included a group that suffered from mixed emphysema and bronchitis. Kanazawa et al. found that sputum of asthmatics and bronchitic COPD patients had higher VEGF concentration than that of normal participants. But VEGF levels were lower in the emphysema group. The mixed emphysema and bronchitis group had the same amount of VEGF in their sputum as normal volunteers (Kanazawa, Asai, Hirata, & Yoshikawa, 2003). They also found a reverse relationship between VEGF and FEV1 in the patients with bronchitis and conversely a positive relationship between them in the emphysema group. Patients with emphysema who had higher VEGF had better gas diffusion. They concluded that increased VEGF in bronchitis and reduced VEGF in emphysema are related to airway obstruction and suggested that VEGF may contribute to the pathogenesis in both morphologic groups of COPD, i.e. airway disease and emphysema.

Kanazawa et al.'s study is interesting in that it measured sputum VEGF levels in different subcategories of COPD, i.e. chronic bronchitis versus emphysema, separately. But in reality, only a minority of COPD patients presents as pure emphysema or pure chronic bronchitis.

Kasahara et al.'s research supports Kanazawa et al.'s findings. Kasahara et al. reported less VEGF and VEGFR-2 expression in emphysema compared to normal lung (Kasahara et al., 2001). The same investigators produced emphysema in rats with VEGF receptor blockers (Kasahara et al., 2000).

Koyama et al. assessed broncho-alveolar lavage fluid (BALF) from 11 current smokers and 16 nonsmokers and some patients with interstitial lung diseases. They found reduced VEGF in BALF of smokers with normal lungs compared to nonsmokers with normal lungs and in smokers with interstitial disease of the lungs compared with nonsmokers

with interstitial disease (Koyama et al., 2002). This study suggested that smoking reduces VEGF.

Transforming growth factor- β (TGF- β): TGF- β is present in many tissues and cells in the human body (de Boer et al., 1998). TGF- β can be found in lower respiratory tract fluid of the human lung in a concentration 15-fold higher than the plasma concentration; however, its source in the lungs is not clear yet (Yamauchi, Martinet, Basset, Fells, & Crystal, 1988). Alveolar macrophages, monocytes, lymphocytes, neutrophils, eosinophils, epithelial cells and mesenchymal cells all are potential sources for TGF- β 1 (Assoian et al., 1987; Magnan et al., 1994). Both TGF- β 1 and TGF- β 3 were detected in bronchial epithelium and alveolar macrophages. TGF- β 1 was also found in endothelial cells, vascular smooth muscle cells, bronchial smooth muscle cells, macrophages in the alveoli and interstitium, and as a diffuse stain within the ECM. Three isoforms, TGF- β 1, TGF- β 2 and TGF- β 3 have been recognised. (Coker et al., 1996; Magnan et al., 1994).

TGF- β 1 is a very vital factor for lung homeostasis with a number of intracellular Smads acting as down stream transcription factors. The TGF- β -Smad pathway (see below) plays a role in cell differentiation and apoptosis (Ten Dijke, Goumans, Itoh, & Itoh, 2002). Their activity protected the lungs against emphysema in animal studies (Morris et al., 2003). Mutant mice that had disrupted alleles for TGF- β 1 die after birth (Shull et al., 1992). In addition to counteracting the destructive activity of MMPs, TGF- β 1 also increases elastin production (McGowan, Jackson, Olson, Parekh, & Gold, 1997) (Kucich, Rosenbloom, Abrams, & Rosenbloom, 2002).

TGF- β has angiogenic activity. It induced angiogenesis and stimulated the synthesis of collagen in vivo (Roberts et al., 1986).

Fibroblasts are the main effector cells in tissue remodelling and the main producers of extracellular matrix (ECM) (Hogg & Timens, 2009) (Postma & Timens, 2006). TGF- β regulates cell growth and differentiation (Khalil et al., 1991). Through modulation of

fibroblasts, TGF- β is one of the regulators of ECM components including collagen in COPD (de Boer et al., 1998). TGF- β regulated transcription of IL-1 and increased its

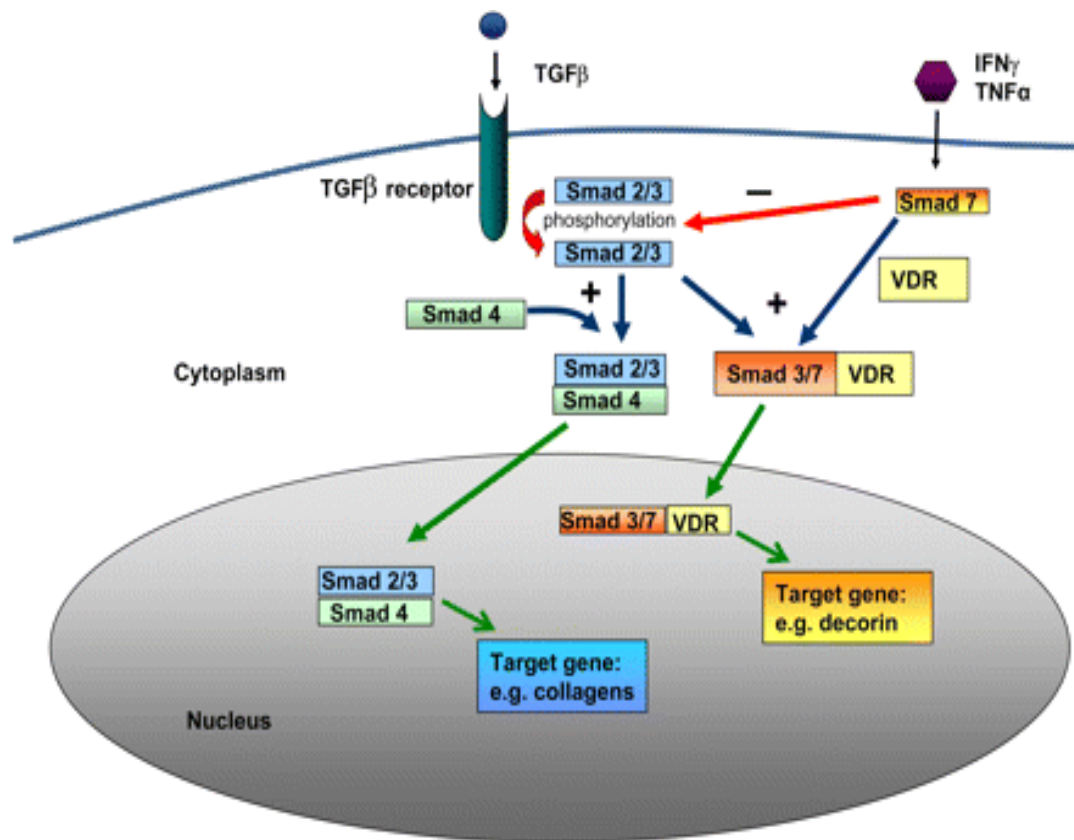


Figure 2-17. Schematic representation of the main components of the Smad pathway.

Smad pathway mediates TGF- β signals intracellularly Smad 2 and Smad 3 make a complex after activation of TGF- β receptors and interact with Smad 4 which is a transporter. Gene transcription starts after this complex finds way into the nucleus. These components of Smad shuttle between nucleus and TGF- β receptors. Smad 7, that is activated by TNF- α and other proinflammatory cytokines, is an inhibitor of activation of Smad 2 and Smad 3. The TGF- β -Smad complex regulates transcription of ECM proteins, MMP and tissue inhibitor of MMP. TGF = transforming growth factor; VDR = vitamin D receptor. (Source: (Postma & Timens, 2006)).

production by monocytes. IL-1 is a regulator of fibroblasts proliferation (Wahl et al., 1987).

TGF- β receptors are suggested to play an important role in the pathogenesis of COPD through their function on regulating Smad pathways (Hogg & Timens, 2009) (Figure 2-17). The TGF- β -Smad complex regulates transcription of ECM proteins, MMP and TIMP (Itoh, Itoh, Goumans, & Ten Dijke, 2000) {Ten Dijke, 2004 #244} (Zandvoort et al., 2006) (Schiller, Javelaud, & Mauviel, 2004).

A study by Vignola et al. reported that TGF- β was increased in the epithelium and the lamina propria of subjects with bronchitis and to a lesser extent in asthma compared to controls. In this study, BB taken from 19 subjects with chronic bronchitis (seven had COPD) were compared with asthma subjects and thirteen nonsmoking healthy subjects. COPD subjects had at least a 30 pack-year smoking history. There were positive correlations between the number of epithelial cells stained for TGF- β /mm of BM and the number of cells positive for TGF- β /mm² of LP (the authors incorrectly described the subepithelial area as submucosa in their article) with Rbm thickness in the asthma and chronic bronchitis groups (Vignola et al., 1997). This study did not find any difference between the study groups in the peripheral airways.

Another group of investigators, however, found higher bronchiolar and alveolar TGF- β 1 expression in 14 subjects with COPD compared to 14 controls who underwent lung resection for lung cancer (de Boer et al., 1998). COPD patients were all either current or former smokers with moderate to severe disease. The authors did not sub-categorise COPD subjects into chronic bronchitis with airway obstruction, chronic bronchitis with out airway obstruction or emphysema. Therefore, clearly the design of the study was different from the Vignola et al.'s study and this may explain the differences in results between them. However, in contrast to de Boer et al., TGF- β 1 and TGF- β receptor type 1 was found to be significantly lower in epithelial cells, stromal cells and type-2

alveolar cells in moderate COPD compared to controls in another study (Zandvoort et al., 2006). In Zandvoort et al.'s study subjects with chronic bronchitis were excluded.

4.j-Antiangiogenic factors:

Antiangiogenic factors have inhibitory effects on angiogenesis. Fifteen inhibitory factors have been recognised. There is a lot of interest in these factors as they can be used potentially in the treatment of cancers. Angiostatin, endostatin, interferon- β , thrombospondin 1, interferon alpha (IFN- α), platelet factor 4, tissue inhibitor of matrix metalloproteins (TIMPs), tumstatin, and angiopoietin-2 are some of these inhibitors. Some of these have been used in clinical trials for cancer treatment (Folkman, 2004) (Gerber, McMurtrey et al., 1998) (Puxeddu et al., 2005). Some factors have negative effects on VEGF production, including endostatin, angiostatin and TIMPs.

Endostatin is an anti-angiogenic factor. Endostatin is the C-terminal fragment of collagen XVIII and is produced by cleavage of collagen XVIII by proteolytic enzymes (Distler et al., 2003; O'Reilly et al., 1997). It targets angiogenesis regulatory genes and inhibits the proliferation, migration and tubule formation of endothelial cells (Asai et al., 2002; Bischof et al., 2009; Folkman, 2006; F. Yan, Huadong, Yujie, Nan, & Yongzhang, 2009). It also increases apoptosis and downregulates VEGF in tumor cells (Distler et al., 2003). For antiangiogenic effects, endostatin needs to bind to its receptors to enter the cell. A number of molecules including matrix metalloproteinase 2 (MMP-2) are considered as its receptors. As said before, in the process of angiogenesis, endothelial cells need to migrate through the disintegrated BM and MMP-2 disintegrates the BM. Endostatin makes a complex with MMP-2 to block its proteolytic function (Y.-M. Kim et al., 2000). Nucleolin acts as a shuttle protein for endostatin and translocates it into the nuclei of endothelial cells. Endostatin blocks endothelial cell proliferation by inhibiting phosphorylation of nucleolin in the cell nuclei. Nucleolin is a ubiquitous protein with many functions and is a crucial factor for cell proliferation (Shi et al., 2007). It has many functions in cell proliferation including modification of nucleolar chromatin (Monique, et al., 1988). Nucleolin is only present on cell surface of angiogenic vessels, thus mature cells do not present this protein on their surface (F. Yan et al., 2009).

Tumstatin is the noncollagenous 1 domain (NC1) of $\alpha 3$ chain of collagen IV. As said before, collagen IV is a component of the BM. Tumstatin needs MMP-9 proteolytic activity to separate from α chain of collagen IV and imply its antiangiogenic activity (Burgess et al., 2010; Hamano et al., 2003). Tumstatin down regulation has been suggested as a marked feature of asthmatic airways, but was reported as normal in COPD tissues (Burgess et al., 2010). This study indicated that tumstatin inhibited endothelial cell tube formation and endothelial cell proliferation.

4.k-Inflammation:

Exposure of the lungs to noxious gases, toxic particulates and environmental pollution triggers the inflammatory cascade and provokes the production of proinflammatory cytokines. These cytokines that are produced in the lungs can release into the blood and produce systemic reactions, like the synthesis of acute phase reactants by the liver. Alveolar macrophages produce many inflammatory cytokines after exposure to environmental pollution (Hogg & Timens, 2009) (van Eeden et al., 2001). Chronic obstructive lung disease is characterized by *inflammatory changes* in airway mucosa. This inflammation persists even after quitting (Wright et al., 1983) (Rutgers et al., 2000) (Willemse et al., 2005). In one study on COPD subjects, inflammation remained longer than 3.5 years after smoking cessation in BB (T. S. Lapperre et al., 2006).

Although studies have constantly found evidence of inflammation in the airways of COPD subjects and also in smokers without airway obstruction, the type of the cells varied in different reports. Neutrophils, macrophages, B-cells and CD8 T-cells having been reported in the inflammatory infiltrate in sputum, lavage fluid or biopsy specimens (Bosken, Hards, Gatter, & Hogg, 1992; Chung & Adcock, 2008; Di Stefano et al., 1998; Hunninghake & Crystal, 1983; Leopold & Gough, 1957; Linden et al., 1993; Retamales et al., 2001; Saetta et al., 1997; Totti, McCusker, Campbell, Griffin, & Senior, 1984). Some studies also reported increased eosinophils and mast cells in COPD (Fujimoto, Kubo, Yamamoto, Yamaguchi, & Matsuzawa, 1999; Grashoff et al., 1997; Lacoste et al., 1993; Pesci et al., 1994).

Yudong Wen who obtained her PhD in 2007 in our group, studied the inflammatory characteristics in the airways (sputum, BAL and biopsies) of asymptomatic smokers with normal lung function (number = 31, 14 current smokers and 17 ex-smokers) and COPD subjects (number = 34, 17 current smokers and 17 ex-smokers) and compared them to healthy nonsmoking controls (number = 30). The study results are summarised below (currently submitted to International Journal of COPD) (Y. Wen, 2007):

1. Smokers with normal lung function versus nonsmoking controls:
 - a. Total cells and macrophages (the major cell population) were significantly increased in sputum of smokers with normal lung function. These changes were more intense in current smokers with normal lung function than in ex-smokers with normal lung function.
 - b. Mast cells were significantly increased in sputum of asymptomatic ex-smokers.
2. COPD versus asymptomatic smokers with normal lung function:
 - a. Lymphocytes and eosinophils were significantly increased in sputum from COPD.
 - b. There was a trend for the presence of greater number of neutrophils in the sputum of ex-smokers with COPD compared to ex-smokers with normal lung function, but the trend did not reach statistical significance ($p = 0.06$).
 - c. Ex-smokers with COPD had higher total sputum cells compared to ex-smokers with normal lung function.
 - d. COPD subjects had higher neutrophils, lymphocytes and eosinophils as percents of total cells in sputum compared to smokers with normal lung function.
3. Current smoker COPD versus ex-smoker COPD:
 - a. Reduced neutrophils in sputum
 - b. Increased total cell count, macrophages and eosinophils% (as percent of total cells) in BAL
 - c. Reduced lymphocytes% and neutrophils% in BAL
 - d. No difference in biopsy cellularity

One of the features of Wen's study was great care was taken to exclude those with bacteria "colonization" in the airways, which itself may stimulate an inflammatory response; this is not true of most studies published as far as we can tell.

Sputum neutrophilia has been shown in multiple studies (Chung & Adcock, 2008; Keatings, Collins, Scott, & Barnes, 1996). Sputum neutrophil count, expressed as a percent of total cells in induced sputum, was higher in stage II-IV COPD subjects compared to healthy nonsmokers, healthy smokers and mild COPD in O'Donnell et al.'s study. Neutrophil count had a negative correlation with FEV1% predicted (O'Donnell et al., 2004). Another study found more neutrophils in sputum of smokers and ex-smokers with airway obstruction than smokers without airway obstruction and also more neutrophils in sputum of those who had chronic sputum production than those who do not. Sputum neutrophilia was correlated with rapid airflow decline in longitudinal assessment of the subjects (Stanescu et al., 1996). Ronchi et al. examined either spontaneously or induced expectorated sputum in COPD and normal subjects and found increased neutrophils as a percent of total cells in the COPD group compared to the control group (Ronchi et al., 1996).

Rutgers et al. compared 18 ex-smokers with COPD to 11 healthy controls and studied their sputum, BAL and BB for inflammation. Ex-smokers had quit for at least 6 months. COPD subjects had more inflammatory cells of various types than controls (Rutgers et al., 2000). The number of CD8+ T-lymphocytes was increased in the small airways from smokers with chronic bronchitis and COPD compared to a control group with normal lung function (Saetta et al., 1998). Airway T-cells in COPD expressed CXCR3 and produced INF- γ ; i.e. they were Th1 type of lymphocytes (Panina-Bordignon et al., 2001). (CXCR3 is a chemokine receptor that together with CCR5 is expressed mainly on Th1 lymphocytes; it regulates leukocyte trafficking). Grumelli et al. reported that both CD4+ and CD8+ cells in peripheral lung tissue of COPD subjects showed the characteristics of TH1 lymphocytes and suggested that TH1 cells are linked to tissue destruction (Grumelli et al., 2004). Alveolar macrophages (AMs) from the lungs of smokers were increased in numbers and have altered characteristics compared to

nonsmokers (Rasp, Clawson, Hoidal, & Repine, 1978). Bronchial lavage from patients with “chronic bronchitis” with or without airway obstruction showed increased level of myeloperoxidase and eosinophilic cationic protein compared to normal subjects (Riise et al., 1995). (Myeloperoxidase and eosinophilic cationic protein are indicators of increased activity of PMN and eosinophils respectively).

A study reported more eosinophils and neutrophils in sputum of subjects with emphysema (diagnosed with lung function, HRCT of the lungs and gas diffusion) compared to normal healthy volunteers (Fujimoto et al., 1999). The eosinophil number reduced with prednisone. In contrast, there was no change in neutrophil count.

Airway neutrophilia, at least in some compartments of the airway wall, has been reported in many articles. A study examined lobar bronchi using resected samples from 18 smokers, 9 with COPD and 9 asymptomatic with normal lung function. Subjects with clinical chronic bronchitis and chronic airflow limitation had an increased number of neutrophils and macrophages and a decreased CD4/CD8 ratio in the bronchial glands compared to asymptomatic subjects. All specimens were resected because of localised pulmonary lesions. The number of neutrophils was also increased in the epithelium. There was no difference in the number of eosinophils and mast cells in the two groups (Saetta et al., 1997).

Di Stefano et al. reported significantly greater numbers of neutrophils, macrophages and natural killer lymphocytes in subepithelium of BB from smokers with severe airway obstruction compared to smokers with normal lung function. FEV1 was inversely correlated with the number of inflammatory cells in their study (Di Stefano et al., 1998).

A group found more neutrophils in BAL fluid of COPD subjects compared with those with chronic bronchitis without airway obstruction (Lacoste et al., 1993). The number of neutrophils in BB was not different between groups.

A study compared small airways for the number of inflammatory cells in specimens from 16 COPD patients and 15 subjects without COPD all undergoing lung resection for

treatment of lung cancer. There were both current- and ex-smokers in both study groups. The investigators examined the specimens for mast cells, macrophages, neutrophils, eosinophils, T-cells and B-cells. They found more mast cells and macrophages in the epithelium of bronchioles, but not other areas, in smokers with airway obstruction. The average number of cells in the epithelium was calculated per mm of basement membrane and for other parts of the tissue as per square mm of the surface area examined. There was no relationship between the number of epithelial mast cells and bronchodilator (tested by salbutamol) reversibility. They did not find any difference in neutrophil and T-cell numbers; only few B-cells and eosinophils were detectable (Grashoff et al., 1997).

Summary: Many studies have shown sputum neutrophilia in sputum from COPD subjects (Chung & Adcock, 2008; Keatings et al., 1996). A suggestive correlation between sputum neutrophilia and airway obstruction has been reported (O'Donnell et al., 2004; Stanescu et al., 1996). Studies that examined biopsy tissue have reported various types of inflammatory cells. Neutrophils were reported to be increased in BB from subjects with chronic bronchitis with severe airflow obstruction compared to smokers with normal lung function (Di Stefano et al., 1998). Small airways were reported to have more lymphocytes in COPD (Di Stefano et al., 1998; Saetta et al., 1998). Yudong Wen, a PhD graduate from our group, found that total cells and macrophages were increased in sputum of smokers with normal lung function compared to nonsmoking controls and lymphocytes and eosinophils were significantly increased in sputum of COPD subjects compared to smokers with normal lung function (Y. Wen, 2007). Examining the same biopsies that were later used in my study, Dr. Wen did not find any difference in cellularity between current- and ex-smokers with COPD.

4.1-Cigarette smoke and inflammation:

Cigarette smoke contains a lot of oxidants (Pryor & Stone, 1993). Oxidative stress directly damages cells and tissue components and induces mucus production by epithelial cells and is toxic to epithelial cilia (Hilbert & Mohsenin, 1996, 9-11; Repine et al., 1997) (Figures 2-4 & 2-18).

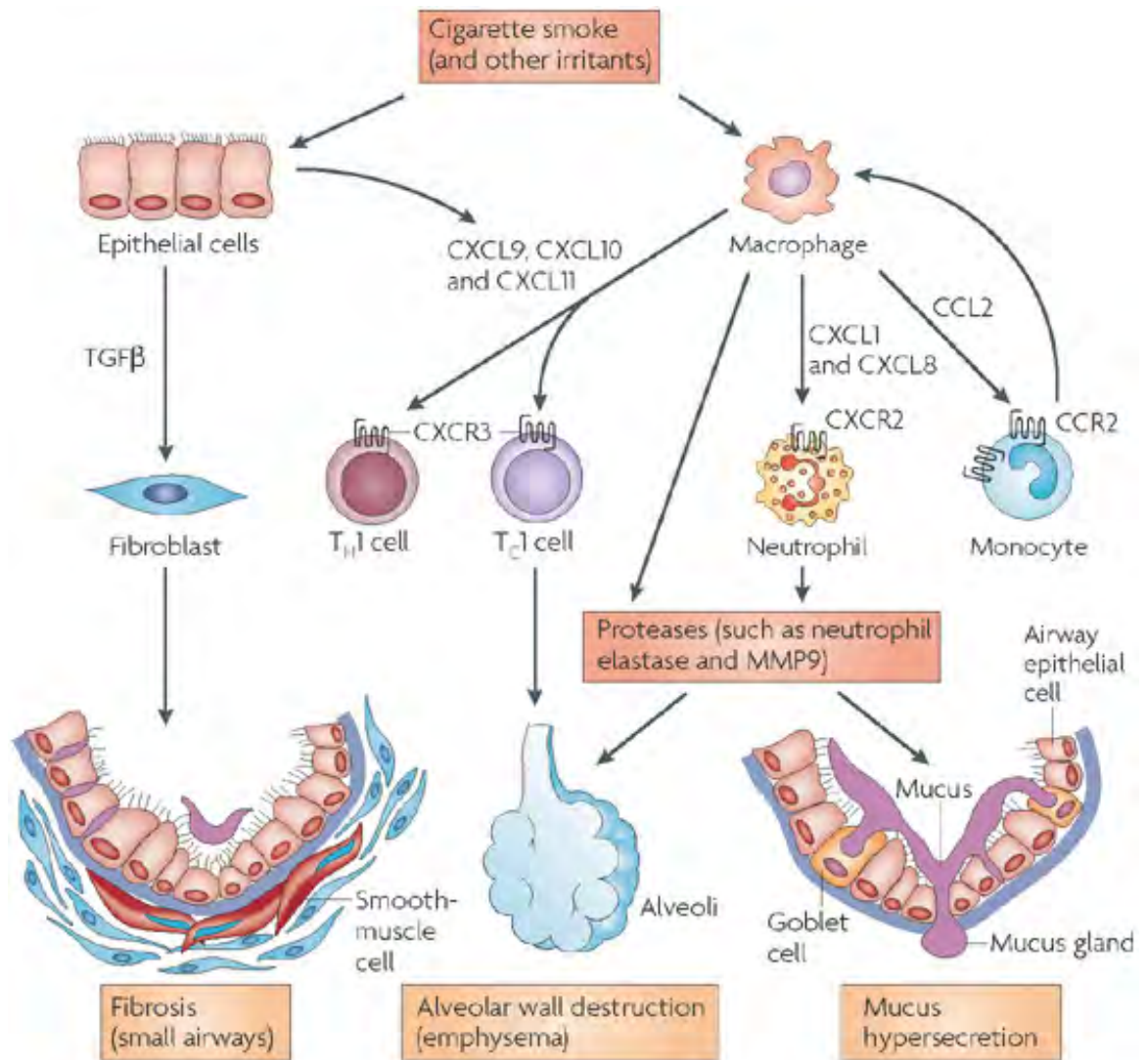


Figure 2-18. *Pathogenic effects of cigarette smoke.* The activated epithelial cells and macrophages attract inflammatory cells to the tissue. The result of inflammatory cell recruitment is alveolar destruction in the parenchyma and mucus hypersecretion in airways. $\text{TGF-}\beta$ released by the epithelial cells and macrophages stimulates fibroblasts and promotes fibrosis. (Source: (P. J. Barnes, 2008)). [CC = chemokines or chemotactic cytokines. CC chemokines have two adjacent cysteine amino acids near their amino terminus. CXC chemokines have two N-terminal cysteines separated by another amino acid]. [CCR and CXCR = receptors to these chemokines].

Oxidants increase nuclear factor- κ B (NF- κ B) activity which increases the transcription of inflammatory genes (see below). Histone acetyltransferase, activated by oxidative stress increases the activity of multiple inflammatory genes by allowing increased access of transcription factors to relevant DNA (P. J. Barnes et al., 2003).

Oxidative stress also activates PI3K which may play a crucial role in multiple inflammatory pathways. It has been postulated that PI3K activation by oxidative stress induces histone deacetylase (HDAC2) reduction, which alongside to other mechanisms, has been suggested as being the cause of relative corticosteroid insensitivity in COPD by some authors (see Section 13. Glucocorticosteroids) (P. J. Barnes & Adcock, 2009; To et al., 2010).

4.m-Intracellular regulators of inflammation:

Transcription factors: Production of inflammatory mediators is controlled by transcription factors.

Function:

- Transcription factors modulate gene transcription and production of inflammatory molecules such as cytokines, adhesion molecules, inflammatory enzymes and inflammatory receptors. These factors play key roles in cell growth and differentiation.

Mechanisms of action:

- Inactive NF- κ B remains in the cell cytoplasm as its prototype p50/p65 NF- κ B and bound to inhibitor of κ B (I- κ B). When a stimulator activates the NF- κ B signal pathway, I- κ B is phosphorylated by I- κ B kinase 2 (IKK-2) and removed. This results in the release of the p65 nuclear localisation sequence and translocation of NF- κ B into the nucleus
- Transcription factors bind to the promoter region of target genes to change the rate of gene transcription
- Activated NF- κ B binds to the κ B recognition sites on inflammatory genes

- Activated NF- κ B can also bind to coactivators, such as cyclic AMP response element binding protein (CBP), which causes histone acetylation and thus activates gene expression (Figure 2-19).

Stimulators of transcription factors: Examples of stimulators of transcription factors are:

- Tumor necrosis factor-alpha (TNF- α)
- IL-1 β
- Viral protein
- Oxidative stress

Regulation of function: There are a number of mechanisms through which transcription factor activity is modulated, over and above their own rate of production. For example:

- Transcription factors can interact with each other. These interactions influence the way that each factor can regulate the transcription of a specific gene
- The activity of transcription factors is regulated by cell surface or cytosolic receptors. Some transcription factors are seen in different types of cells and some are cell-specific
- Glucocorticosteroids inactivate coactivators of NF- κ B (see Section 13, Glucocorticoids, Mechanisms of glucocorticoid action later) (P. J. Barnes, 2006; P. J. Barnes & Adcock, 2009; McKay & Cidlowski, 1999).

RAGE: The Receptor for Advanced Glycation End-products (RAGE) belongs to the immunoglobulin superfamily. This receptor takes its name from its ligands AGE (Advanced Glycation End-products of Proteins) classically known to be produced by hyperglycemia but now known to be much more complicated than this and produced also by other proinflammatory stimuli including oxidative stress in particular. AGEs are the products of reduction of proteins, lipids or nucleic acids by adding reducing saccharide derivatives to these compounds (Nienhuis et al., 2009; Oldfield et al., 2001). This occurs during normal ageing and is exaggerated in diabetes mellitus and in the

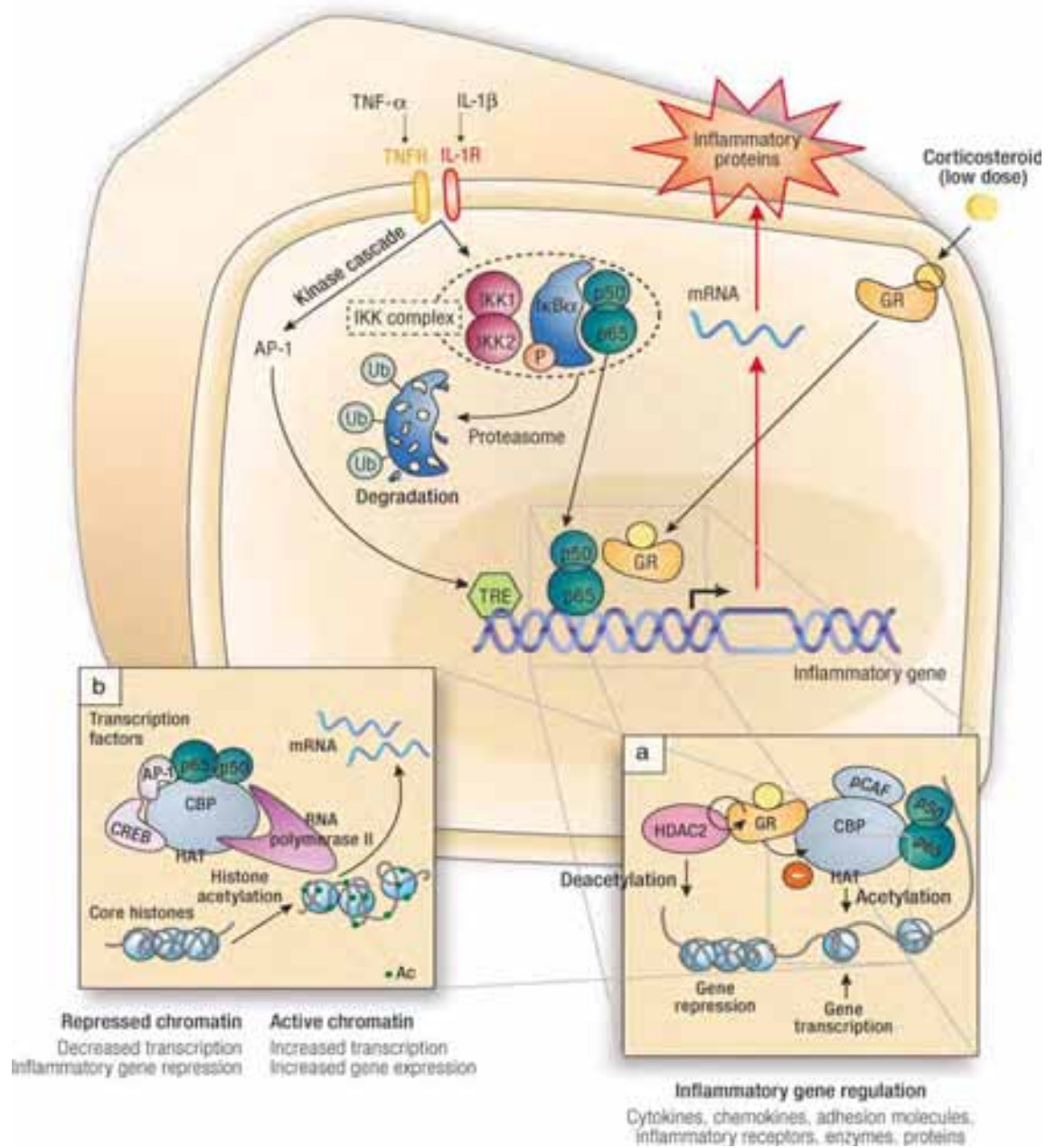


Figure 2-19. Please see next page for legend.

Legend to **Figure 2-19. *Transcription factors***. Molecules like tumor necrosis factor alpha (TNF- α) and IL-1 β inactivate NF- κ B inhibitor (I- κ B α) after binding to their specific cell receptors. Then active units of NF- κ B (P50 and P65) translocate to the nucleus and increase transcription of inflammatory genes.

Inset a: In the cell nucleus active units of NF- κ B bind to molecules with histone acetyl transferase (HAT) activity such as CREB-binding protein (CBP) or p300/CBP-activating factor (PCAF). Acetylation of histone protein results in increased expression of inflammatory proteins. Glucocorticoids activate their receptor which then translocates to the nucleus and inhibits HAT and recruits HDAC2. HDAC2 reverses histone acetylation and therefore suppresses inflammatory genes.

Inset b: CREB-binding protein (CBP) has HAT activity. It acetylates histone and allows binding of RNA polymerase II.

CREB = Cyclic AMP response element binding protein, NF- κ B = Nuclear factor- κ B, AP-1 = activator protein-1 (Source: (P. J. Barnes, 2006))

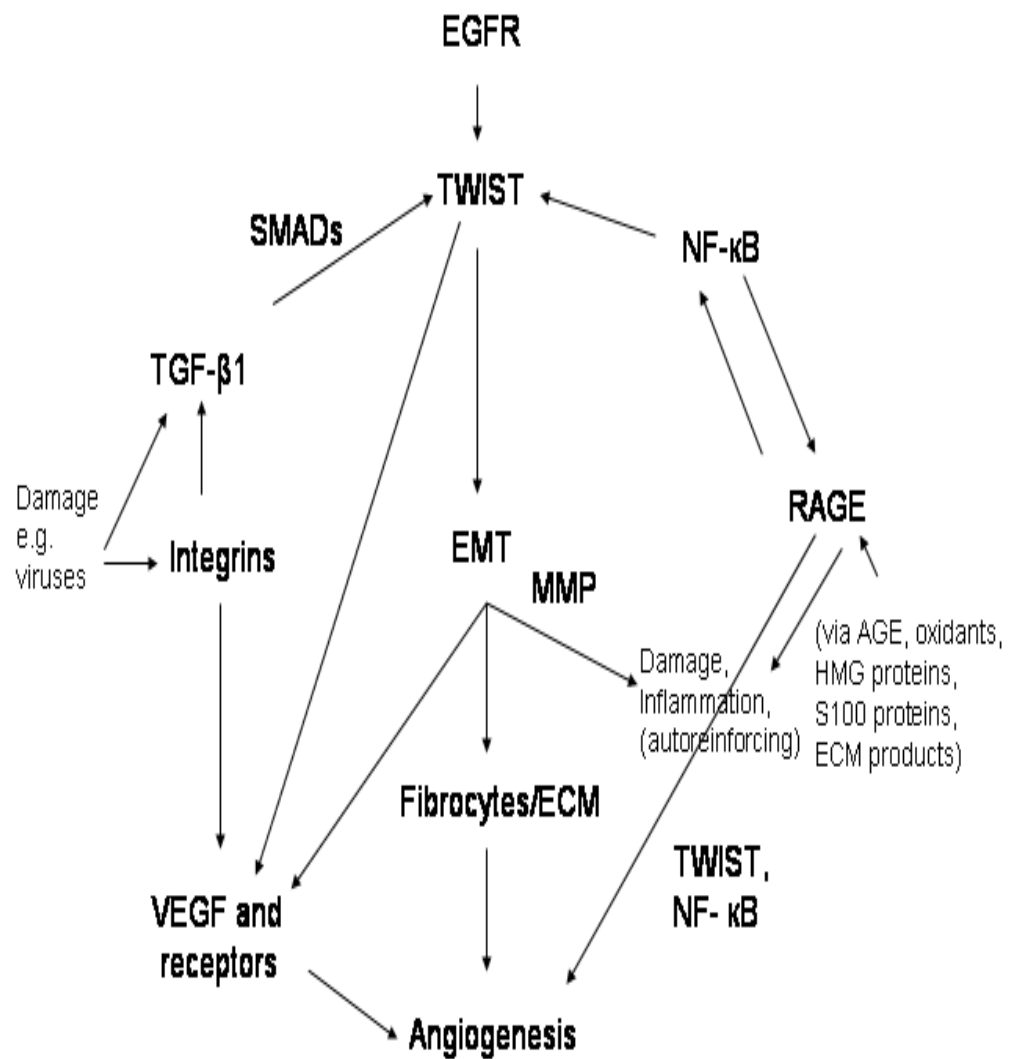


Figure 2-20. Please see next page for legend.

Legend to **Figure 2-20**. *Potential inter-relationship among Transcription factors, inflammation, EMT and angiogenesis.* RAGE, NF- κ B, TGF- β and VEGF plot the relationship between inflammatory transcription factors, EMT and angiogenesis. AGE and other ligands bind to RAGE and activate intracellular transcription factors such as NF- κ B. NF- κ B increases transcription of inflammatory genes and therefore production of inflammatory mediators. ROS (reactive oxygen species) delivered by cigarette smoke, environmental pollution and inflammatory cells induce NF- κ B and RAGE activities and therefore inflammation. Activated RAGE and ROS upregulate the production of TGF- β which provokes EMT changes in cells. Twist, a transcription factor which has a key role in EMT, can be stimulated by EGFR, NF- κ B and TGF- β . VEGF which is an angiogenic factor is upregulated by Twist. Pathological conditions cause BM disruption and induce the production of TGF- β and EMT.

AGE = Advanced glycation end-products, BM = Basement membrane, EGFR = Epithelial growth factor receptor, EMT = epithelial-mesenchymal transition, HMG = High motility groups, NF- κ B = Nuclear factor- κ B, RAGE = Receptor for AGE, ROS = Reactive oxygen species, S100 proteins = A group of proteins which are 100 percent soluble in ammonium and are present in inflammatory environment, TGF- β = Transforming growth factor beta, VEGF = Vascular endothelial growth factor. Both NF- κ B and Twist are transcription factors.

presence of oxidative stress. AGEs accumulate in the extracellular matrix (ECM) and are present in inflamed tissues. They activate several receptors including RAGE and affect the function of cells (Nienhuis et al., 2009). There is close interaction between RAGE and the Toll-receptor system, thus overlapping with microbial inflammatory stimulation. [Toll-like receptors (TLR) are a class of proteins that recognise microbial antigens when they penetrate normal barriers of the body, such as skin or mucous membranes, and stimulate the immune system].

Interactions between AGE and RAGE activate intracellular mechanisms of cytokine production including TGF- β and IL-1 (Figure 2-20). AGE stimulate the expression of vascular cell adhesion molecule-1 (VCAM-1), Intercellular adhesion molecule-1 (ICAM-1), E-selectin, VEGF, monocyte chemotactic protein-1 (MCP-1), MMP and several other ILs which are involved in inflammatory diseases (Nienhuis et al., 2009; Oldfield et al., 2001).

RAGE is expressed constitutively in normal cells and its expression substantially increases in inflammatory conditions and with binding of TNF- α , AGE and other ligands. Binding of ligands to RAGE also generates ROS (reactive oxygen species), which in turn activates NF- κ B and therefore transcription of inflammatory genes. This is one of a number of self-propagating reinforcement mechanisms associated with the RAGE system (Nienhuis et al., 2009) (Morbini et al., 2006; Sparvero et al., 2009) (Figures 2-19 & 2-20).

In addition to AGE, other ligands such as HMG (high mobility groups) and S100 proteins (100% soluble in ammonium) can activate RAGE. Activation of multiple intracellular mediators such as NF- κ B is a consequence of RAGE activity (Nienhuis et al., 2009) (Sparvero et al., 2009).

HMG are nuclear non-histone chromosomal proteins. An archetype of these proteins, HMGB1, activates RAGE by forming physico-chemical complex with other pro-inflammatory compounds because of its highly charged structure (Sparvero et al., 2009).

S100 proteins are a variety of compounds that are prevalent in inflammatory diseases. S100A12 binds to RAGE and has chemotactic activity for mast cells (W. X. Yan et al., 2008). S100A12 and S100B can activate structural and inflammatory cells through RAGE and increase the expression of pro-inflammatory cytokines. S100A4 functions both in intracellular and extracellular environments. Its exact mechanisms of action are not clear yet but S100A4 is involved in a wide range of biological processes. Intracellular S100A4 interacts with cytoskeleton structures and extracellular S100A4 mediates many cellular responses including motility, survival and differentiation. S100A4 is reportedly related to a wide variety of diseases in several body organs including pulmonary diseases. These diseases are characterised by inflammatory and fibrotic phenomena mainly associated with remodelling and epithelial-mesenchymal transition (EMT) (Schneider, Hansen, & Sheikh, 2008).

The lungs are exposed to environmental pollution and smoke which induces recurring injury to the airway epithelium. The lungs are sites of intense oxidative activity in airways and parenchyma as a result of smoking, air pollution and inflammatory diseases. Oxidative damage to the lungs may activate AGE production with subsequent activation of RAGE and the inflammatory mechanisms that were described above (Morbini et al., 2006). COPD is also a chronic inflammatory disease with activation of inflammatory mediators and cells and these RAGE-related mechanisms need much more investigation in this context; it would be a good candidate for the ongoing and apparently self-sustaining inflammation that occurs in COPD especially after quitting cigarettes and high susceptibility to viral infection-induced exacerbations.

4.n-Inflammatory mediators and epithelial-mesenchymal transition (EMT):

(Figure 2-20). Studies on these intracellular mechanisms may link pro-inflammatory mediators to structural tissue changes. TGF- β that can be activated by AGE-RAGE interaction induces EMT in alveolar epithelial cells. EMT is seen in different pathological conditions in humans such as malignancies or fibrosis of lungs in IPF or in the ischemic kidney. In this process in these “models”, epithelial cells change their characteristics and shape, convert to fibrocytes and migrate within tissue. Epithelial cells

are located on their basement membrane (BM). When pathological conditions result in disruption of the BM, epithelial cells start secreting cytokines such as TGF- β and epithelial growth factor (EGF) and express the markers of EMT. EMT has been seen in response to reactive oxygen species and exposure to AGE. EMT is therefore, a response of epithelial cells to injury in adult tissues (Willis & Borok, 2007) (Pozharskaya et al., 2009) (Kida, Asahina, Teraoka, Gitelman, & Sato, 2007; Zeisberg et al., 2001). What is urgently needed is studies based on these insights into chronic airway disease including COPD. Some data on bronchiolitis obliterans syndrome (BOS) suggest the potential involvement of EMT in this context. BOS as a consequence of allograft lung transplantation is characterised by airway remodelling and extracellular matrix deposition leading to airway obstruction as a result of increased fibroblastic activity. The source of these fibroblasts is not completely clear yet but there is now some evidence supporting EMT as part of this process (Borthwick et al., 2009; C. Ward et al., 2005).

TGF- β is a multifunction cytokine. It affects cell proliferation and differentiation. It also mediates the conversion of epithelial cells to myofibroblasts. Fibroblasts can also acquire myofibroblast characteristics under TGF- β influence. In an in vitro study Oldfield et al. showed AGE induced epithelial mesenchymal transdifferentiation. Cells produced TGF- β when exposed to AGE (Oldfield et al., 2001) (Willis & Borok, 2007; Willis et al., 2005).

A key intermediary transcription factor involved in EMT is Twist (basic helix-loop-helix protein). Twist regulates EMT during embryogenesis and metastasis. Its roles have been shown in lung fibrosis following viral infection and in kidney fibrosis. Hypoxia, epidermal growth factor receptor, TGF- β and NF- κ B are amongst inducers of Twist. Induction of Twist through retroviral infection of a type of renal tubular epithelial cell with highly organized cell to cell adhesions resulted in loss of adhesions, cell scattering and change of morphology to fibroblastic type, i.e. an EMT like reaction. Twist was also able to increase angiogenesis by upregulating VEGF synthesis (Mironchik et al., 2005) (Kida et al., 2007; Pozharskaya et al., 2009) (J. Yang et al., 2004).

Research on the roles of RAGE and other transcription factors in inflammatory mechanisms and potential EMT in large airways in COPD and smokers with normal lung function has never been performed. It is also important to differentiate the mechanisms that lead to disease establishment in smokers, i.e. irreversible airway disease. These subjects are proposed candidates for future research by our group.

4.o-Relationship between inflammation and tissue remodelling:

Examination of the lungs of smokers suggests that the primary lesion in the small airways was likely to be inflammation that resulted in connective tissue deposition in the airway walls (Cosio et al., 1978). Chronic inflammation causes detachment of parenchymal connections to airways and therefore, airway distortion and narrowing (Cosio Piqueras & Cosio, 2001). A relationship between inflammation of small airways and parenchymal destruction in centriacinar emphysema has been shown (Retamales et al., 2001; Saetta, Kim, Izquierdo, Ghezzi, & Cosio, 1994).

Inflammation and abnormal tissue repair or remodelling are tightly bound together in COPD (Hogg & Timens, 2009). Enlargement of mucous glands and increase in proliferating activity of the epithelial cells are linked to inflammation (Hogg & Timens, 2009). Inflammation can cause fibrosis and gland hypertrophy and it can chronically increase smooth muscle tone (Repine et al., 1997). A study on current smokers and ex-smokers showed association of inflammatory cell infiltration in the mucosa of large airways and around glands with symptoms of chronic bronchitis (Mullen et al., 1985). Inflammation, both by increasing deposition of connective tissue and also reducing the supportive traction force of lung parenchyma on small airway wall, causes airflow limitation (Cosio et al., 1978). A study using BB from smokers showed a reverse relationship between FEV1 and severity of inflammatory cell infiltration in the airways (Di Stefano et al., 1998).

Inflammation and angiogenesis are also related. VEGF has chemotactic properties (Postma & Timens, 2006). Macrophages can secrete angiogenic factors under hypoxic conditions and angiogenesis promotes inflammation. Inflammatory cells find access to

the site of inflammation through new vessels and nutrients and oxygen supply is increased to the site because of better blood supply (Jackson et al., 1997).

5-Physiopathology:

COPD is a chronic and largely irreversible disease that is physiologically characterized with airway obstruction. Theoretically, involvement of the large airways, peripheral airways and lung parenchyma can all produce airway obstruction (A. Nagai, West, & Thurlbeck, 1985).

Accompanying to acceleration of annual FEV1 decline in COPD, as classically reported by Fletcher et al. (Fletcher & Peto, 1977), is the reduction of FVC and FEV1/FVC ratio (FER) in this disease (P. T. Macklem, 2010).

Emphysema:

- Reduction of lung parenchymal elastic recoil pressure in emphysema decreases the supporting force around small airways. The result will be early collapsibility of small airways during expiration (Snider, 1985) (Hogg & Timens, 2009)
- Narrowing of small airways causes expiratory flow obstruction, i.e. FER becomes less than normal
- At residual volume (RV), bronchioles are not stretched by elastic fibers anymore and subsequently collapse earlier than normal [RV is the volume of the lungs in deep exhalation; the lungs can not be completely emptied in life despite deep exhalation]
- Therefore, RV is greater than normal; this is referred to as air trapping
- Total lung capacity (TLC) may also increase as a result of emphysema [TLC is the volume of the lungs in deep inhalation. It is the sum of FVC and RV] (Corbin, Loveland, Martin, & Macklem, 1979; P. T. Macklem, 2010; Mead, Takishima, & Leith, 1970)
- When the increase in RV becomes greater than the increase in TLC, FVC reduces (Figure 2-21) (P. T. Macklem, 2010)

- Elastic recoil pressure contributes to the expiratory force needed to exhale alveolar gas (Hogg, 2008). Therefore, driving expiratory force decreases as elastic tissue is destroyed in emphysema.
- Both reduced driving force and early collapsibility of small airways are considered as the underlying mechanisms of airflow limitation in emphysema (Cosio Piqueras & Cosio, 2001).

Airway involvement:

- Some studies suggested that changes in small airway structure may be more important (Hogg, Macklem, & Thurlbeck, 1968) (Hogg, 2004; Van Brabandt et al., 1983; Yanai, Sekizawa, Ohnishi, Sasaki, & Takishima, 1992). Small airway wall thickness and consequently airway lumen narrowing is probably important contributing factor in airflow obstruction in COPD
- Destruction of airway cartilage (chondromalacia) intensifies dynamic compression

Large airway pathology: is related to increased sputum production and impaired mucociliary function. But, it is not as important a predictor for airflow limitation and dyspnea as emphysema and small airway pathology (Hogg, 2004; E. Haydn Walters et al., 2010) (Steven D. Shapiro & Ingenito, 2005). Nevertheless, a group of investigators found an inverse relationship between CD8+ T-cells in large airway walls of COPD subjects and FEV1 (O'Shaughnessy et al., 1997). Another study found a negative relationship between FEV1 in COPD with Reid index (ratio of thickness of mucous gland layer to total thickness of the airway wall) (Berend, Wright, Thurlbeck, Marlin, & Woolcock, 1981). Submucosal glands are heavily infiltrated with neutrophils, and neutrophil elastase is a protein secretagogue (Fahy, Schuster, Ueki, Boushey, & Nadel, 1992; Schuster, Ueki, & Nadel, 1992).

In normal conditions, small airways' contribution to total airway resistance is minor (Hogg et al., 1968; Peter T. Macklem, 1998; P. T. Macklem & Mead, 1967) and large airways are responsible for most of it (Hogg, 2004). But small airway resistance

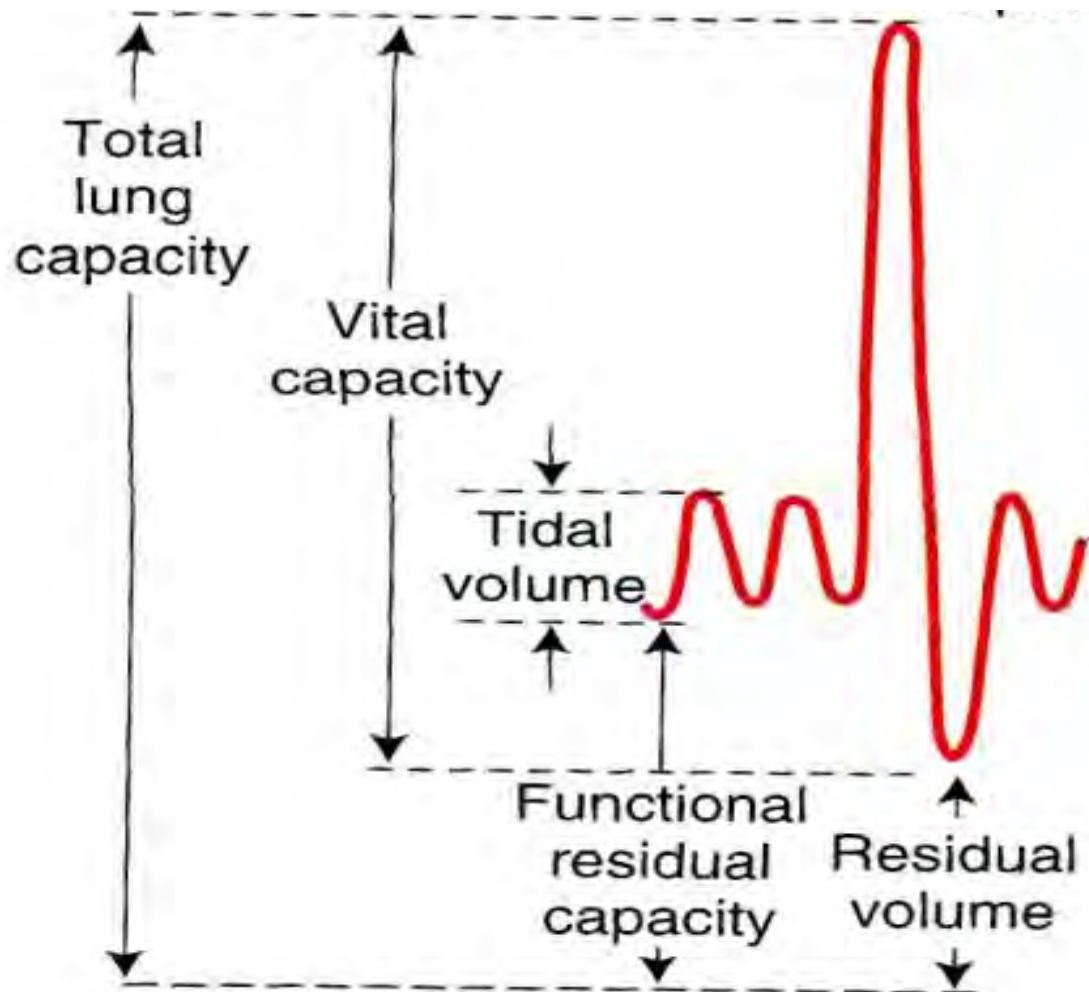


Figure 2-21. Lung volumes. TLC is the total volume of the lungs in deep inspiration and can be calculated as the sum of RV and VC (or FVC). Despite deep expiration, some air remains in the lungs. RV is the volume of the lungs at the end of deep expiration. When air trapping happens, the increase in RV may be greater than the increase in TLC; thus VC (or FVC) reduces. (Source: (West, 2005)).

increases 4 to 40 times with the evolution of COPD and becomes the major part of airway obstruction in COPD (Hogg et al., 1968). Deposition of connective tissue around small airways in emphysema may aggravate airway obstruction by reducing the ability of the airways to dilate during expansion of the lungs in inhalation. In mild emphysema, peripheral airway resistance (small airway resistance) increases but total lung resistance remains stable.

There is an inverse relationship between inflammatory cell infiltration of small airway walls and FEV1 (Hogg et al., 2004). Other studies have confirmed these reports (Van Brabant et al., 1983; Yanai et al., 1992) (Gelb et al., 1993; Hogg et al., 1994; Leopold & Gough, 1957; Wright et al., 1984). A study found parenchymal destruction to be more important than small airway involvement in airflow obstruction (Matsuba & Thurlbeck, 1972).

In a study on the lungs of subjects who died of severe COPD, investigators found a correlation between emphysema and the proportion of bronchioles less than 400 μm in diameter with ante-mortem airflow limitation. (The number of bronchioles less than 400 μm in diameter is expressed as a percentage of the total number of bronchioles) (A. Nagai, West, Paul et al., 1985). Emphysema was negatively related to BDR and small airway involvement was related to dyspnea, arterial CO₂ tension and expiratory flow limitation and increased residual volume. Quite interestingly, increase in muscle and fibrosis in bronchiolar walls were beneficial, as these changes were related to better arterial oxygen tension, lower arterial CO₂ tension, higher flow rates and lower rates of right sided heart abnormalities. Therefore, the investigators proposed that increased muscles and fibrosis of the wall of bronchioles help them keep their shape and remain open (A. Nagai, West, & Thurlbeck, 1985).

7-Clinical manifestations:

COPD presents predominantly with progressive shortness of breath over years. It starts slowly and is progressive and disabling in advanced stages. Cough that may produce

phlegm is seen in many patients (chronic bronchitis). Chronic bronchitis may precede airway narrowing by many years (GOLD, 2007) (S. Guerra et al., 2009). However, in a study in Denmark, investigators did not find a relationship between chronic bronchitis and progression towards COPD (Jorgen Vestbo & Lange, 2002).

Patients with COPD may suffer from systemic symptoms like muscle wasting, anorexia, anemia and fatigue (GOLD, 2007) (Hogg & Timens, 2009) (A. Nagai, West, & Thurlbeck, 1985; Emil F. M. Wouters, Creutzberg, & Schols, 2002). The mechanisms of this is poorly understood but presumed to be the result of cytokines such as TNF- α “leaking” from inflamed lung tissue into the circulation. Exposure of the lungs to noxious gases, toxic particulates and environmental pollution triggers the inflammatory cascade. Alveolar macrophages produce many inflammatory cytokines after exposure to environmental pollution (Hogg & Timens, 2009) (van Eeden et al., 2001). Circulating levels of IL-1 β (interleukin- 1 beta), IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF) were high after acute air pollution exposure (van Eeden et al., 2001). Tumor necrosis factor alpha (TNF- α) and IL-1 are two important cytokines that are involved in local and probably systemic inflammation. TNF- α is produced in a dose-response manner after exposure to air pollutants (van Eeden et al., 2001). These two cytokines provoke febrile reactions through stimulation of the hypothalamus, stimulate the liver to produce acute phase reactants (e.g. CRP) and the bone marrow to produce and release leukocytes.

8-Diagnosis:

COPD is diagnosed by a history of exposure to noxious particles, clinical manifestations and lung function study showing airflow obstruction that is not completely reversible (Figure 2-7). There should be an absence of typical asthma features.

9-Acute exacerbations:

During the course of COPD, patients experience aggravation of symptoms that may need more intensive treatment than usual daily management. Exacerbations are defined as an acute change in the patient's baseline symptoms beyond day-to-day variability sufficient

to warrant a change in management (B. R. Celli et al., 2004). Most exacerbations are due to viral or bacterial infections (Quon, Gan, & Sin, 2008; Sethi, 2000).

10-Complications:

COPD may be complicated during its course with acute exacerbations, respiratory failure, pulmonary hypertension, right sided heart failure, cardiac arrhythmias, muscle wasting, pneumothorax and pulmonary embolism (Ueng et al., 2000) (Yeh, DeGuzman, & Kramer, 2002) (Castaldi, Hersh, Reilly, & Silverman, 2009) (Lee, Yap, Pek, & Keong Ng, 2004).

11-Management:

COPD is now defined as a treatable disease (GOLD, 2007) (Bartolome R. Celli, 2008). The therapeutic approach differs according to the severity of disease. Principally treatment of COPD includes smoking cessation, bronchodilators (especially anticholinergics), inhaled corticosteroids (especially where acute exacerbations are frequent), oxygen for patients with significant chronic hypoxemia, rehabilitation and in a few patients, surgical interventions (Bartolome R. Celli, 2008). Lung transplant is an option in some COPD patients with end stage disease that are otherwise almost fit. Corticosteroids in the treatment of COPD will be discussed in more details later.

12-COPD versus asthma:

Asthma and COPD are both common chronic airway diseases (K. R. Chapman et al., 2006; Masoli et al., 2004). They may coexist in some people who have a background of asthma and cigarette smoking (Stefano Guerra & Martinez, 2009). It seems that investigators have tried to test different hypotheses in COPD that had previously been tested in asthma. It therefore seems reasonable to discuss why this order of “COPD after asthma” has happened and argue why it is not quite right to think of these two diseases as simply just two different variations of chronic inflammatory airway diseases.

- Aetiologies of the two diseases are quite different: hypersensitisation in asthma but exposure to smoke and dust in COPD (P. J. Barnes, 2008; GOLD, 2007).

- Age of onset is different in asthma and COPD: COPD is typically a disease of middle age and the elderly (Masoli et al.) (GOLD, 2007; Lugogo, Que, Fertel, & Kraft, 2010).
- Asthma is recognised clinically with the triad of episodic dyspnea, wheezes and coughing. COPD starts insidiously and progresses with time (GOLD, 2007; Lugogo et al., 2010; Masoli et al.).
- Lung parenchymal destruction (emphysema) is seen in COPD but not in asthma (Bergeron & Boulet, 2006; Peter T. Macklem, 1998; A. Nagai, West, Paul et al., 1985; Steven D. Shapiro & Ingenito, 2005).
- The inflammatory characteristics of these two diseases are different: mainly (but not always) eosinophilic in asthma but neutrophilic or lymphocytic in COPD (Bergeron & Boulet, 2006; Gibson, 2009).
- There is enough convincing evidence to assume that airway remodelling in COPD is different from that in asthma (Bergeron & Boulet, 2006). Hypervascularity of the LP and thickening of the Rbm are characteristically reported in asthma (Boulet et al., 1997; Dunnill, 1960; Bryce N. Feltis et al., 2006; Jeffery et al., 1992; Li & Wilson, 1997; Orsida et al., 1999; J. W. Wilson & Li, 1997) but not constantly in COPD (Chanez et al., 1997; Jeffery, 2004; Liesker et al., 2009).
- Our expectations from treatment in these two diseases are different: an almost normal life in most patients with asthma but, slowing down deterioration and helping patients stay independent for their daily life tasks as long as possible in severe COPD. (P.J Barnes, 1998) (P. J. Barnes, Pedersen, & Busse, 1998) (P.M.A. Calverley et al., 2006).

- Pulmonary hypertension, cor pulmonale and right heart failure are seen in end-stage COPD but not in classic asthma (Ueng et al., 2000) (Yeh et al., 2002) (Castaldi et al., 2009) (Barbera, Peinado, & Santos, 2003; Lee et al., 2004) (Traver, Cline, & Burrows, 1979) (Kessler, Faller, Fourgaut, Menecier, & Weitzenblum, 1999; Kessler et al., 2001).

Therefore, these two diseases can overlap but also have major differentiating features. In clinical practice they are usually easy to differentiate, but there are some patients that cause diagnostic dilemmas, especially chronic asthmatics that smoke (Kandane-Rathnayake et al., 2009).

13-Glucocorticosteroids:

13.a- Mechanisms of action:

Steroids have been used since the late 1940s as antiinflammatory drugs (De Bosscher, Vanden Berghe, & Haegeman, 2003) and inhaled formulations have been available for over 40 years. Inhaled glucocorticosteroids (ICS) are universally used as the first line long-term treatment in asthma management for anything more than mild intermittent disease (P.J Barnes, 1998) (Orsida et al., 1999) (P. J. Barnes et al., 1998). Especially when combined with long-acting β -agonists (LABA), corticosteroids suppress airway inflammation and reverse remodelling changes in asthma. They affect airway inflammation in COPD but there are many fewer studies, especially relating to remodelling in smoking-related disease. Their therapeutic effects are by genomic and nongenomic mechanisms (Figure 2-22):

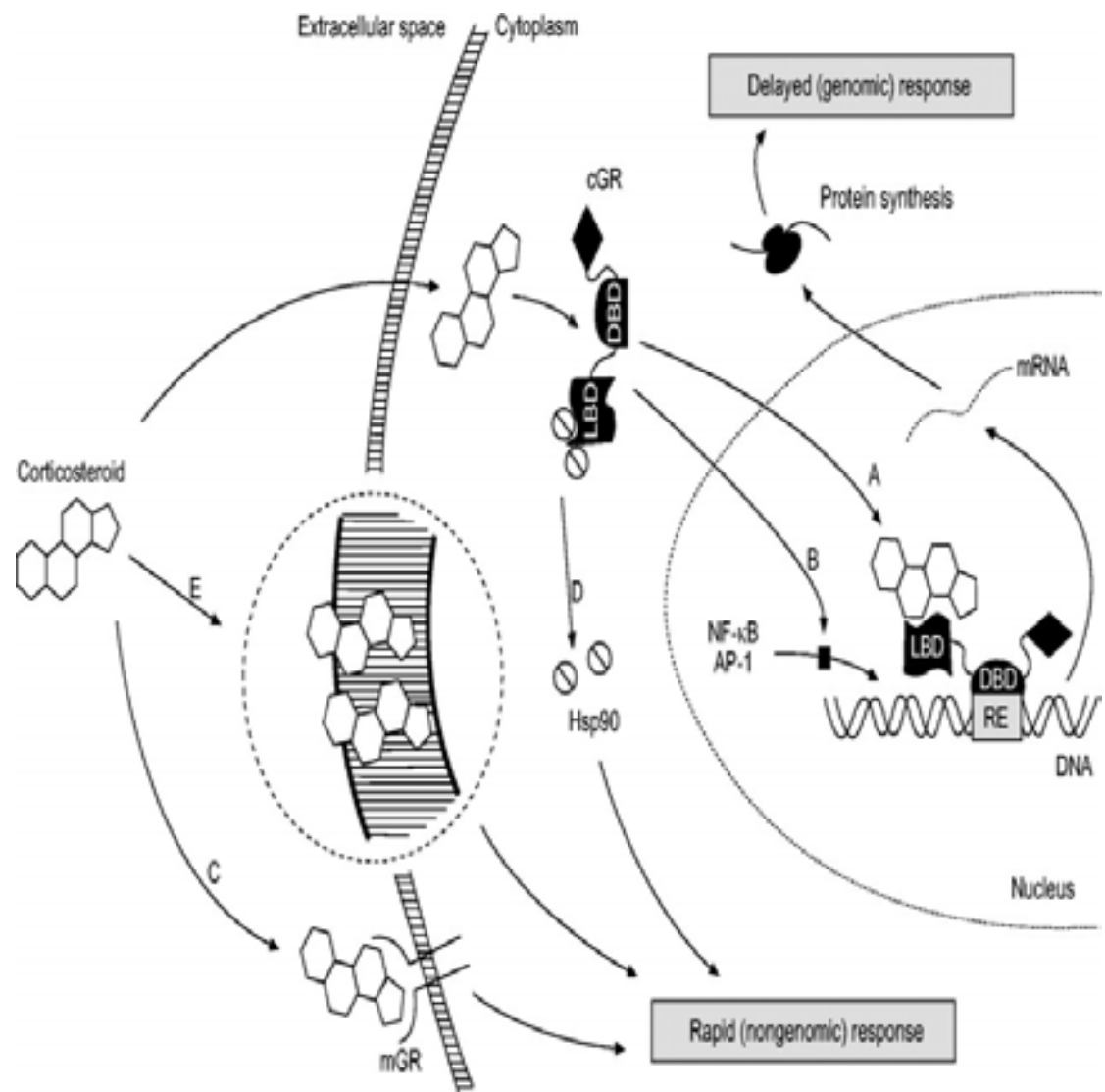


Figure 2-22. *Intracellular mechanisms of corticosteroid receptor function.* Steroids bind to cell receptors and translocate to the nucleus to influence transcription either by binding to DNA or by inactivating transcription factors such as NF-κB. The figure also shows non-genomic mechanisms of action of corticosteroids. cGR: cytoplasmic glucocorticoid receptor; mGR: membrane glucocorticoid receptor; LBD: ligand-binding domain; DBD: DNA-binding domain; Hsp90: heat-shock protein 90; RE: response element; NF- κB: nuclear factor- kappa B; AP-1: activating protein-1. (Source: (G. Horvath & Wanner, 2006)).

- The nongenomic functions are rapid and take effect in seconds to minutes. These mechanisms are not related to alterations of gene expression. Nongenomic functions are mediated by receptors on the cell membrane and within caveolae (Mendes, Pereira, Danta, Duncan, & Wanner, 2003; Norman, Mizwicki, & Norman, 2004; Rhen & Cidlowski, 2005; Thio et al., 2001a; C. S. Watson & Gametchu, 2003). Caveolae are membrane invaginations that can be found in the mucosal blood flow and protection against exercise-induced bronchoconstriction in children.

Vascular tone alterations occur within minutes but reduction of airway blood flow needs almost 30 minutes to reach its maximal effect after ICS (G. Horvath & Wanner, 2006). The mechanism by which locally administered steroids cause vasoconstriction is not related to noradrenalin release from nerve endings but it is probably mediated by enhancing noradrenalin physiological effect, probably by blocking noradrenaline uptake at nerve endings. Steroids acutely inhibit noradrenalin uptake by non-neuronal cells in the nerve endings in bronchial smooth muscle cells and thus, increase norepinephrine concentration at α -adrenergic receptor sites (Gabor Horvath, Lieb, Conner, Salathe, & Wanner, 2001; G. Horvath & Wanner, 2006; J. A. Russell & Kircher, 1985).

Vasoconstrictor effects of ICS were more marked in asthmatic subjects than nonasthmatics (Kumar, Brieva, Danta, & Wanner, 2000), perhaps because they have more vessels, but there are no comparable data in COPD.

A study showed that airway mucosal blood flow reduced in minutes following inhalation of three different types of ICS in both healthy and asthmatic subjects (Mendes et al., 2003). Another study showed that a single dose of ICS had a protective effect against exercise induced asthma in children (Thio et al., 2001b).

- Corticosteroids diffuse across the lipid bilayer of the cell membrane to bind to glucocorticoid receptors (GRs), in the cell cytoplasm. GRs are transcription factors and belong to the family of steroid hormone receptors. GRs have five

serine residues that are phosphorylated under a variety of conditions by cycline-dependent kinases and mitogen-activated protein kinases (MAPK). The pattern of phosphorylation of these serine residues provides distinct transcriptional activity to GRs. For example, when GRs are phosphorylated at serine 203 they are inactive in the cytoplasm, and when phosphorylated at serine 211 they activate DNA transcription. GRs are bound to chaperone and co-chaperone molecules in their inactive form in the cytoplasm. After binding to glucocorticoids, they activate and move into the nucleus and bind to glucocorticoid response elements as the upstream transcription regulatory parts of genes. The resulting complex then either facilitates or inhibits gene transcription by using coactivator or corepressor proteins.

- NF- κ B persists in the cell cytoplasm in an inactive form bound to an inhibitory protein named inhibitor of κ B (I- κ B). I- κ B specifically binds to NF- κ B making a trimer that can not bind to DNA. Phosphorylation of I- κ B releases NF- κ B. NF- κ B translocates into the nucleus and binds to DNA sequences known as NF- κ B elements. NF- κ B also binds to coactivators such as CBP (cyclic AMP response element binding protein) or PCAF (p300/CBP-activating factor). CBP and PCAF have histone acetylase activity (HAT). This results in histone acetylation, opening up of chromatin and its access to RNA polymerase II resulting in gene transcription. Glucocorticoid-GR complex binds to coactivators to directly inhibit HAT activity and recruits HDAC2 to reverse histone acetylation and thus to suppress inflammatory genes (P. J. Barnes & Adcock, 2009; De Bosscher et al., 2003; G. Horvath & Wanner, 2006; McKay & Cidlowski, 1999; Rhen & Cidlowski, 2005) (Figures 2-19 & 2-21).
- It also has been suggested that PI3K, activated by oxidative stress, reduces HDAC2 (P. J. Barnes & Adcock, 2009; To et al., 2010).
- GRs also interact with other intracellular transcription factors such as NF- κ B (Black, Oliver, & Roth, 2009). Investigations suggest that GR and NF- κ B physically interact as mutual transcription antagonists. In human cells NF- κ B has

been shown to enhance the expression of a wide variety of genes such as cytokines, adhesion molecules and complement factors. Many of the genes involved in inflammatory cytokine production, despite being repressed by GR, do not present negative glucocorticoid response elements. Therefore, mechanisms other than gene repression should be responsible for the inhibitory effect of GR. It has been suggested that NF- κ B and GR physical interaction is the mechanism of inhibitory function of glucocorticoids in these circumstances. For example, it has been shown that p65 subunit of NF- κ B and GR can interact (McKay & Cidlowski, 1999; Rhen & Cidlowski, 2005).

- Glucocorticoids switch on the activity of MAPK phosphatase 1 gene. This enzyme dephosphorylates and inactivates MAPK pathways. Kinases such as MAPK have a crucial role in the activation of inflammatory pathways in cells. MAPK mediate transcriptional and post-transcriptional changes in gene expression in response to pro-inflammatory stimuli (Adcock, Caramori, & Chung, 2008; P. J. Barnes & Adcock, 2009; Lasa, Abraham, Boucheron, Saklatvala, & Clark, 2002).
- Finally, Glucocorticoid-GR receptor complex suppresses the production of prostaglandins via three mechanisms, i.e. induction of annexin-1, activation of MAPK phosphatase-1 and repression of transcription of cyclooxygenase-2 (Rhen & Cidlowski, 2005). Prostaglandins are produced by the enzymatic action of cyclooxygenase on arachidonic acid and have bronchoconstrictive and inflammatory properties.

1- Cytosolic phospholipase A2 α , activated by inflammatory mechanisms, induces the release of arachidonic acid and its metabolites such as prostaglandins. Annexins are regulators of a variety of intracellular functions including inflammation. Inhibition of phospholipase A2 by annexin-1 (lipocortin-1) has been reported in many studies. Glucocorticoids induce annexin and thus block the

release of arachidonic acid and its metabolites (S.-W. Kim et al., 2001; Rhen & Cidlowski, 2005).

- 2- Inflammatory mechanisms such as viral or bacterial infections and inflammatory cytokines activate MAPK cascades resulting in the activation of inflammatory and immune genes. As already stated, glucocorticoids induce MAPK phosphatase 1 which dephosphorylates and inactivates all members of the MAPK family proteins and therefore inhibits inflammatory gene transcription. MAPK phosphatase 1 also blocks the phosphorylation of cytosolic phospholipase A2 α by MAPK (Lasa et al., 2002; Rhen & Cidlowski, 2005).
- 3- NF- κ B stimulates the transcription of cyclooxygenase 2, and the binding of glucocorticoid-GR complex to NF- κ B switches this power off (McKay & Cidlowski, 1999; Rhen & Cidlowski, 2005).

13.b-Use of ICS in Asthma:

The effect of ICS in asthma is explained very briefly, as it is beyond the subject of this thesis. The next section will address the use of ICS in COPD in more detail.

ICS are the drug of choice in long-term control of active asthma (P.J Barnes, 1998; GINA, 2009) (Orsida et al., 1999) (P. J. Barnes et al., 1998; Cameron, Cooper, Crompton, Hoare, & Grant, 1973; Field, Jenkinson, Frame, & Warner, 1982; Hajiro & Ishihara, 2001; Meltzer, Kemp, Orgel, & Izu, 1982; Welch, Levy, Smith, Feiss, & Farrar, 1997). They also control inflammation in asthma airways (Bentley et al., 1996; Jeffery et al., 1992; Olivieri et al., 1997; C. Ward et al., 2002); and have been reported to have effects on airway remodelling (Asai et al., 2003; Chetta et al., 2003; B. N. Feltis et al., 2007; Foschi, Castoldi, Corsi, Radaelli, & Trabucchi, 1994; Olivieri et al., 1997;

Orsida et al., 1999; Todorova, Gurcan, Miller-Larsson, & Westergren-Thorsson, 2006; C. Ward et al., 2002).

13.c-Use of ICS in COPD:

Compared to asthma, the response to ICS in COPD is not as dramatic and it has been suggested that it is limited to some subgroups of the disease. In fact, some authors are against the use of ICS in COPD (Suissa & Barnes, 2009) (Rodrigo, Castro-Rodriguez, & Plaza, 2009). However, their use in COPD has become very popular among clinicians and patients (Rodrigo et al., 2009). According to GOLD guidelines, ICS are mainly recommended for use in patients with severe COPD to decrease the frequency of exacerbations (GOLD, 2007). Investigators have assessed the effects of ICS on clinical, physiological and inflammatory manifestations of COPD. ICS, used alone or in combination with LABA, have been compared either with placebo or with other drugs that are frequently a part of treatment in COPD. In this section I first cite the articles that assessed the effects of ICS on clinical manifestations and physiological parameters. Then I will cite articles that have assessed ICS on airway inflammation in COPD. There are very few data about the effects of ICS on airway remodelling in COPD (E. Haydn Walters et al., 2010) that will be reviewed briefly in the final part of this section.

13.c.1-Clinical response

(Table 2-1): Reports that compared ICS alone with placebo or other drugs are reviewed first, followed by the reports that evaluated combinations of

ICS with long-acting beta agonists (LABA) or other medications. Finally, two studies on the consequences of withdrawal of ICS are presented. Table 2-1 has summarised all these studies.

A randomised, double blind and multinational study of 281 outpatient COPD subjects in Europe, South Africa and New Zealand was published in 1998 (Paggiaro et al., 1998). Subjects (with a history of chronic bronchitis, mean FEV1% predicted ~ 57%, FEV1% predicted range 35-90%, BDR<15%, 50% ex-smokers) were given ICS, fluticasone

propionate (FP, 500 µg twice daily for 6 months). The effect of FP was compared with placebo. The number of subjects that had at least one exacerbation was not significantly different between groups. Moderate to severe exacerbations occurred less often in the

ICS group and the difference was significant. Diary PEF, FEV1 and FVC during treatment and a 6-minute walk test at the end of the treatment were significantly better on ICS than placebo. Cough and sputum improved significantly compared to placebo.

In another large study (Inhaled Steroids in Obstructive Lung Disease in Europe, ISOLDE), investigators assessed the effects of long-term ICS on lung function, exacerbations and health status in 751 patients with moderate to severe COPD (Burge et al., 2000). Patients were randomised to take either 500 µg twice daily of fluticasone propionate (FP) or placebo for 3 years. Before the trial, all patients were treated with 2 weeks of prednisolone, 0.6 mg/kg/daily to see if their response to a short-course prednisone could predict response to ICS. Main outcomes were the annual rate of decline in FEV1 and frequency of exacerbations. This study did not find any significant difference in the annual rate of decline between FP and placebo. Nevertheless, post-bronchodilator FEV1 remained higher in the FP group than placebo throughout the study. Exacerbation rate significantly reduced in the FP group compared to the placebo group. Health status, assessed by SGRQ deteriorated significantly less rapidly with FP than placebo. The effects of ICS were independent of the initial response to oral prednisolone. The conclusion was FP was not effective in slowing the rate of decline of FEV1, but provided better FEV1 than placebo, slowed down the rate of health deterioration and reduced the rate of exacerbations. Therefore, the authors believe that their study supported the use of FP in moderate to severe COPD patients.

Table 2-1. Summary of published studies on clinical responses to ICS in COPD*

Study group	Drugs	Methods	Main results
(Paggiaro et al., 1998)	FP 500 µg /bd x 6 months vs. placebo	RCT, multicenter 281 subjects Hx of chronic bronchitis Mean (range) FEV1% predicted: 57 (35-90) No BDR	FP vs. placebo: Better symptom scores. Reduction of moderate to severe AE. Greater PEF, FEV1 and 6MWT.
(Burge et al., 2000) (ISOLDE)	FP 500 µg /bd x 3 years vs. placebo	RCT 751 subjects Oral prednisone 2 weeks before the trial Moderate to severe COPD	FP vs. placebo: No difference in annual decline of FEV1. Better QOL. Reduced AE. Higher PBD-FEV1. Response to FP not related to initial oral steroid response.
(Pauwels et al., 1999) (EUROSCOP)	Budesonide 400 µg /bd x 3 years vs. placebo	RCT 1277 subjects who continues smoking mild COPD	Budesonide vs. placebo: Less median 3-year FEV1 decline. Higher PBD FEV1 in the first 6 months. More skin bruising.
The Lung Health Study Research Group (2000) (LHS)	Triamcinolone 600 µg/bd x 40 months vs. placebo	RCT 1116 subjects Mean (range) FEV1% predicted 67 (30-90)	Budesonide vs. placebo: Better clinical control. Reduced BHR. Reduced bone density.

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Table 2-1. Summary of published studies on clinical responses to ICS in COPD*
(continued)

Study group	Drugs	Methods	Main results
(Verhoeven et al., 2002)	FP 500 µg vs. Placebo x 6 months	Mild to moderate COPD BDR<10% 23 subjects	FP vs. placebo: No effect on BHR. Stabilised FEV1. Increased FEV1/FVC ratio. Increased maximal expiratory flow rates.
(J. Vestbo et al., 1999)	Budsonide 800/400 µg x 6 months followed by 400 µg/bd up to 3 years vs. placebo	RCT 290 subjects mild to moderate COPD BDR and response to 10-day prednisolone<15%	Budesonide vs. placebo: No improvement in symptoms. No change in annual decline of FEV1.
(I. A. Yang, Fong, Sim, Black, & Lasserson, 2007)	ICS vs. Placebo	Cochrane systematic review 47 studies (RCT) 13139 participants Significant BDR was not an exclusion criteria	ICS vs. placebo: Slight increase in FEV1 in 2-6 months. No decrease in FEV1 rate of decline after 6 months. No effect on mortality. Reduction of the rate of AE. Decreased rate of decline in QOL.
(E. F. M. Wouters et al., 2005)	FP	RCT 497 subjects with COPD were on SFC x 3 months then randomised to: SFC or salmeterol alone	SFC vs. salmeterol: Deterioration of FEV1 and FEV1/FVC ratio in the salmeterol group.
(Choudhury et al., 2007)	FP vs. Placebo	Subjects in primary care Mean FEV1% predicted 55 260 subjects with 8-year Hx of ICS use all stopped their ICS and then randomises to: FP 500 µg/bd or placebo x 1 year	FP vs. placebo: Were less likely to return to their ICS. Fewer AE. Longer time to the first exacerbation.

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Table 2-1. Summary of published studies on clinical responses to ICS in COPD*
(continued)

Study group	Drugs	Methods	Main results
(Peter M. A. Calverley et al., 2007) (TORCH study)	SFC 50/500 µg vs. FP 500 µg vs. Salmeterol 50 µg vs. Placebo All bd x 3 years	RCT International (42 countries) 6112 subjects moderate to severe COPD supported by GSK	SFC vs. placebo: decreased mortality in unadjusted analysis, adjusted analysis p=0.052. SFC vs. other groups: Better QOL. Lower ratio of severe/moderate AE. Higher FEV1. FP vs. placebo: Reduced annual rate of AE. Better QOL. Groups on FP or SFC: Higher 3-year probability of pneumonia.
(Mahler et al., 2002)	SFC 50/500 µg vs. FP 500 µg vs. Salmeterol 50 µg vs. Placebo x 24 weeks	RCT 691 subjects mean FEV1% predicted = 40 Hx of chronic bronchitis ~half of subjects had BDR>12% + 200ml increase in FEV1	SFC vs. all others: Better improvement in dyspnea. Greater increase in pre-dose FEV1. Greater increase in PEF. FP vs. placebo: Better improvement in dyspnea. Greater increase in post-dose FEV1.
(P. Calverley et al., 2003)	SFC 50/500 µg vs. FP 500 µg vs. Salmeterol 50 µg vs. Placebo 1 year	Multinational study 1465 subjects	All groups of active treatment vs. placebo: Reduced severe AE. Increased FEV1. SFC vs. each single therapy: Improved lung function. SFC vs. other treatments: Reduced need for BD.

Continues next page

Table 2-1. Summary of published studies on clinical responses to ICS in COPD*
(continued)

Study group	Drugs	Methods	Main results
(Rodrigo et al., 2009)	ICS + LABA vs. LABA alone	Systematic review of 18 RCTs 12446 subjects	No difference in death. No clinically significant improvement in lung function. ICS + LABA vs. LABA: Increased risk of pneumonia.
(Hanania et al., 2003)	SFC 50/250 µg vs. FP 250 µg vs. Salmeterol 50 µg vs. Placebo All bd x 24 weeks	RCT Multi-center 723 subjects moderate to severe COPD mean BDR 20% in >50% of participants Hx of chronic bronchitis	SFC vs. salmeterol or placebo: Greater pre-dose FEV1. SFC vs. FP or placebo: Greater post-dose FEV1. SFC vs. placebo: Improved symptoms and QOL.
(Szafranski et al., 2003)	Budesonide + formoterol (BF) 160/4.5 µg vs. Budesonide 200 µg vs. Formoterol 4.5 µg vs. Placebo All bd x 12 months	RCT 812 moderate to severe COPD mean FEV1% predicted 36 Supported by AstraZeneca	BF vs. formoterol or placebo: Reduced severe AE. BF vs. placebo: Reduced mild AE. BF vs. placebo or budesonide: Increased FEV1. Reduced BD use. BF vs. others: Increased PEF. Budesonide vs. placebo: Reduced mild AE. Increased FEV1. Increased PEF.

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Table 2-1. Summary of published studies on clinical responses to ICS in COPD*
(continued)

Study group	Drugs	Methods	Main results
(P. M. Calverley et al., 2003) (TRISTAN)	Budesonide + formoterol (BF) 200/4.5 µg vs. Budesonide 200 µg vs. Salmeterol 4.5 µg vs. Placebo All bd	RCT 1022 subjects Stage III and IV COPD Mean ± SD BDR < 6 ± 6% 2 weeks of prednisolone 30 mg and salmeterol before trial	BF vs. others: Fewer withdrawals. Prolonged time to the first AE. Increased FEV1. Better QOL. Increased PEF. BF vs. placebo or formoterol: Fewer AE. Budesonide vs. placebo: Improved QOL.
(L. Nannini, Cates, Lasserson, & Poole, 2004)	Combination of ICS plus LABA vs. placebo	Cochrane systematic review 6 studies (RCT) 4118 participants	ICS/LABA vs. placebo: Reduced rate of AE. Improved QOL. Improved lung function.
(L. J. Nannini, Cates, Lasserson, & Poole, 2007b)	Combination of ICS plus LABA vs. LABA alone	Cochrane systematic review 10 studies (RCT) 7598 participants with severe COPD	ICS/LABA vs. LABA: Reduced AE. Improved QOL. Increased rate of pneumonia.
(L. J. Nannini, Cates, Lasserson, & Poole, 2007a)	Combination of ICS plus LABA vs. ICS alone	Cochrane systematic review 7 studies (RCT) 5708 participants with severe COPD Poor BDR	ICS/LABA vs. ICS: Fewer withdrawals Reduced the rate of AE. Reduced mortality rate. Improved QOL. Improved lung function.

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Table 2-1. Summary of published studies on clinical responses to ICS in COPD*
(continued)

Study group	Drugs	Methods	Main results
(Wedzicha et al., 2008)	SFC 50/500 µg/bd vs. Tiotropium 18 µg/d	RCT Multinational 1323 subjects GOLS III and IV stages	SFC vs. tiotropium: Less mortality. Lower rate of withdrawal from the study.
(Aaron et al., 2007)	Tiotropium + placebo (TP) vs. Tiotropium + salmeterol (TS) vs. Tiotropium + SFC (TSFC) For one year	RCT 449 subjects >40% of subjects in TP and TS leaved the study or used open-label ICS	Tiotropium + SFC vs. tiotropium + placebo: No difference in AE. Improved FEV1. Improved QOL. Reduced hospitalisation for AE or all- cause.
(Singh, Brooks, Hagan, Cahn, & Connor, 2008)	Tiotropium + SFC (TSFC) vs. Tiotropium 18 µg vs. SFC 50/500 µg All bd x 13 weeks	Randomised triple cross- over 41 subjects	TSFC vs. other groups: Improved airway conductance. Reduced IC. Improved dyspnea. Reduced need for BD.

* AE = Acute exacerbations, bd = Twice daily, BD = bronchodilator, BDR = Bronchodilator responsiveness, FP = Fluticasone propionate, Hx = history, LABA = Long-acting β-adrenergic agonists, 6MWT = Six-minute walk test, PBD = post-bronchodilator, QOL = Quality of life, RCT = Randomised clinical trial, SFC = Salmeterol plus fluticasone combination

Pauwels et al. compared budesonide (an ICS), 400 µg twice daily with placebo in a three-year randomised controlled trial in 1277 patients with mild COPD who continued to smoke (European Respiratory Society Study on Chronic Obstructive Pulmonary Disease, EUROSCOP). The median three-year decline of FEV1 was significantly lower with budesonide compared to placebo (140 ml vs. 180 ml). In the first six months of the study, the post-bronchodilator FEV1 improved in the budesonide group but reduced in the placebo group; the difference between the two groups was significant. From nine months to the end of the treatment, FEV1 declined in a similar way between the two groups. The investigators did not find any significant difference between the two groups in side effects of treatment, except for skin bruising that happened more frequently with budesonide (Pauwels et al., 1999).

In a multi-center study conducted by The Lung Health Study Research Group (LHS), 1116 participants with mild to severe COPD (mean post-bronchodilator FEV1% predicted ~ 67%, range 30-90%) were recruited. Triamcinolone (an ICS) 600 µg twice daily for 40 months was compared with placebo. The rate of decline in the post-bronchodilator FEV1 was not significantly different between the two groups, but those on triamcinolone had significantly fewer symptoms and fewer visits to their doctors for respiratory complaints. Methacholine challenge test showed significantly lower reactivity in the active arm. However, the study also showed significant decrease of bone density in the active treatment group after three years compared to placebo. Therefore, the research group concluded that the advantages of ICS in COPD should be weighed against the adverse effects (TLHSRG, 2000).

The effect of ICS (FP, 500 µg twice daily for 6 months) was compared to placebo on bronchial hyper-responsiveness (BHR) in 23 COPD subjects (mild to moderate disease, BDR<10%) (Verhoeven et al., 2002). ICS did not have any effect on BHR. In contrast to the placebo group that showed a steep decline in FEV1, the FP group had a stable FEV1. FEV1/FVC ratio and maximal expiratory flow rates increased significantly with FP compared to placebo.

A 3-year random placebo controlled study assessed the effects of budesonide (800 µg morning and 400 µg evening for 6 months followed by 400 µg twice daily) on lung function and symptoms in 290 patients with mild to moderate COPD in Copenhagen (J. Vestbo et al., 1999). The recruitment criteria included FEV1 response less than 15% to bronchodilators or 10-day course of oral prednisolone. The study did not find any difference in FEV1 decline or respiratory symptoms between the active and placebo arms. These authors questioned long-term use of ICS in mild to moderate COPD.

A Cochrane Review has evaluated randomised controlled trials which have studied the effects of ICS in stable COPD. Forty-seven studies with 13139 participants were reviewed (I. A. Yang et al., 2007). All these studies compared a type of ICS with placebo. Bronchodilator responsiveness was not an exclusion criterion. The results showed that medium term of use (2-6 months) resulted in mild improvement of FEV1. Longer than 6 months of ICS use did not significantly reduce the rate of decline in FEV1. Mortality rate was not different between treatment and placebo groups. The mean rate of exacerbations reduced with ICS. ICS also slowed the decline in quality of life measured by SGRQ. The study could not evaluate the risk side effects from long-term use of ICS. Therefore, they suggested that doctors should balance risk of topical side effects of ICS (thrush and hoarseness) against minor benefits to make decision about long-term use of ICS in their COPD patients.

Two European studies reported withdrawal effects following stopping ICS in patients with COPD.

A multi-center study in the Netherlands reported that withdrawal of FP in 497 COPD patients, even if salmeterol was continued, resulted in statistically significant deterioration of lung function measured as FEV1 and FEV1/FVC ratio. All patients were on salmeterol plus FP combination (SFC) for 3 months and then randomised to either continuing SFC or taking only salmeterol (E. F. M. Wouters et al., 2005). This strongly suggests that ICS do have an effect over and above that of LABA.

Another study in the U.K. evaluated withdrawal of ICS from COPD patients in primary care. 260 COPD subjects with a median 8 years history of ICS use stopped their ICS and then randomised to two groups, either FP 500 µg twice daily or placebo for one year. Patients on placebo were significantly more likely to return to their usual ICS medication following exacerbation. The risk of exacerbation was higher in the placebo group during the trial and this group experienced exacerbations earlier than the FP group. The authors concluded that patients with even mild COPD are at increased risk of exacerbations after withdrawal of ICS (Choudhury et al., 2007). In my opinion, the subjects suffered from more than mild disease, given that the mean value for FEV1% predicted for patients in each group was ~ 55%.

The studies below used combinations of ICS with other medications to compare with placebo or other compounds. Table 2-1 has summarised all these studies.

A large-scale 3-year study, known as TORCH (TOwards Revolution in COPD Health), compared the fluticasone plus salmeterol combination (SFC, 50/500 µg), FP alone (500 µg), salmeterol (50 µg) and placebo twice daily in 6112 moderate to severe COPD patients from 42 countries (Peter M. A. Calverley et al., 2007). [Salmeterol is a long-acting β_2 adrenergic agonist (LABA) acting as bronchodilator and enhancing anti-inflammatory effects of ICS]. The primary outcome was log-rank of time to all-cause death during the 3-year time of the study. Unadjusted log-rank analysis was significantly different in favor of SFC compared to placebo. But adjusted (to take into account an interim analysis) log-rank analyses just missed significance by $p = 0.052$. SFC significantly reduced death compared to FP alone. The authors concluded that SFC is the first intervention since oxygen and smoking cessation to improve survival in COPD (P.M.A. Calverley et al., 2006). The rationale behind the study and its details was published ahead of the final analyses (TORCH, 2004). Health status, assessed by SGRQ (St George's Respiratory Questionnaire), ratio of the severe/moderate exacerbations and improvement in FEV1 were secondary outcomes. SFC was significantly superior compared to placebo, salmeterol alone and FP alone in all three secondary outcomes. FP and salmeterol alone significantly reduced the annual rates of exacerbation, reduced SGRQ score (which means improvement) and increased FEV1 compared to placebo.

During the 3 years of the study, there was no significant difference between groups for drug-related adverse events per year except for 3-year probability of pneumonia that was significantly higher in the groups treated with SFC or FP vs. placebo or salmeterol. The frequency of bone fractures was the same (Peter M. A. Calverley et al., 2007; Bartolome R. Celli et al., 2008). There are some notable points to be made for this study (Rabe, 2007). For example 40% of participants quit the study; withdrawal happened more frequently in the placebo group. This is probably because the subjects on placebo did not tolerate their symptoms and withdraw from the study to get some type of treatment. As the study had a placebo group and lasted for 3 years, patients with high risk of mortality, i.e. patients with frequent exacerbations, might have been excluded. These issues could have resulted in failure of the study to reach an acceptable p value. Although this study failed to prove that combination therapy is the preferred treatment for decreasing mortality, it showed that SFC can improve clinical manifestations and lung function in patients with severe COPD and frequent exacerbations (Rabe, 2007).

Another multicentre, double-blind, controlled trial compared salmeterol plus fluticasone combination (SFC, 50/500 µg twice daily) with salmeterol alone (50 µg twice daily), FP alone (500 µg twice daily) and placebo in 691 subjects with COPD (mean FEV1% predicted 40%) (Mahler et al., 2002). Patients also had chronic bronchitis, i.e. chronic cough and sputum production. Treatment continued for 24 weeks. Primary outcomes were the change in pre-dose FEV1 values with SFC compared to salmeterol and in FP compared to placebo; and change in 2-hour post-dose FEV1 in SFC compared to FP and salmeterol compared to placebo. Other outcomes were morning PEF, the need for albuterol use, dyspnea severity and exacerbation rates. [Albuterol or salbutamol is a short-acting β_2 adrenergic agonist]. At the end of the study the SFC group had a significantly greater increase in pre-dose FEV1 compared to salmeterol, placebo and FP. Throughout the study SFC had significantly greater increase in pre-dose FEV1 compared to salmeterol alone. FP alone had a greater increase in pre-dose FEV1 compared to placebo throughout the study, except for one measurement at week 8. The SFC group had a greater increase in post-dose FEV1 than the placebo and the FP groups. FP alone and salmeterol alone had a greater increase in post-dose FEV1 compared to

placebo. Increase in PEF rate was significantly greater in SFC compared to the other three groups 24 hours after the beginning of the study and stayed greater in the SFC group throughout the study. The severity of dyspnea significantly improved with SFC compared with the other three groups. This improvement started on week one and continued throughout the study. FP also had significant improvement in dyspnea severity compared to placebo at the endpoint. Significant reduction of relief bronchodilator use was observed with SFC compared to placebo and FP. A significant reduction of albuterol use was also observed with each of FP and salmeterol compared to placebo. The authors concluded that SFC provided better improvement in lung function and respiratory symptoms compared to individual compounds and placebo. One very interesting point in this study was that the average BDR in all treatment groups of the study was at least 19%. BDR, defined as an increase of 12% or greater and 200 ml in FEV1, ranged from 51% of subjects in the salmeterol group to 56% of subjects in the placebo group. This degree and frequency of BDR is very untypical for COPD studies.

A multinational study compared salmeterol 50 µg, FP 500 µg, salmeterol plus fluticasone propionate combination (SFC) 50/500 µg and placebo, all twice daily in 1465 COPD patients (P. Calverley et al., 2003). Primary outcome was pre-bronchodilator FEV1 at 12 months of treatment. All methods of active treatment significantly improved FEV1, reduced the number of exacerbations per year and the number of exacerbations that needed oral steroids compared to placebo. The magnitude of treatment effect was higher in patients with FEV1<50% predicted than patients with FEV1>50% predicted. The improvement in lung function was detected at 2 weeks and persisted during the treatment course. SFC significantly improved lung function better than each of the single-therapy groups. SFC significantly reduced the need for rescue use of bronchodilators compared with placebo and salmeterol alone or FP.

One large Systematic Review collated eighteen randomised controlled trials (12,446 participants) that compared LABA alone against combination of ICS plus LABA did not find any difference between the two methods of therapy in overall death and in cardiac or respiratory death (Rodrigo et al., 2009). However, ICS plus LABA combination

showed greater improvement in lung function and in SGRQ total score; but the improvements were not clinically meaningful, i.e. the change in FEV1 was less than 100 ml and reduction in SGRQ was less than 4 (reduction of score indicates improvement). However, ICS plus LABA compared to LABA increased the risk of pneumonia, oral candidiasis and viral infections. Overall the reviewers concluded that the benefits from adding ICS to LABA are at best, very limited.

A multi-center study in the United States recruited 723 patients with moderate to severe COPD to compare the efficacy of FP (250 µg), salmeterol (50 µg), salmeterol plus FP combination (SFC, 50/250 µg) or placebo, all twice daily, for 24 weeks (Hanania et al., 2003). Patients had symptoms of chronic bronchitis. Morning pre-dose FEV1 was compared between the SFC and salmeterol groups and 2-hour post-dose FEV1 was compared between SFC and FP groups. Morning pre-dose FEV1 was significantly better with SFC than salmeterol and placebo and post-dose FEV1 was again significantly better with SFC than FP and placebo at endpoint. SFC compared to placebo also improved symptoms, including shortness of breath, and quality of life. There are surprising similarities in methodology and patient demographics between this study and the study published by Mahler et al. one year earlier (see above) (Mahler et al., 2002), including the characteristics of BDR in the participants. In this study, very much like Mahler et al.'s, the mean BDR was close to 20% in all the study groups and 55% or more of participants in each group were responsive to bronchodilators.

The effects of budesonide plus formoterol (160/4.5 µg twice daily) combination were compared with placebo and each individual drug (budesonide 200 µg, formoterol 4.5 µg, both twice daily) in a 12-months randomised, controlled trial (Szafranski et al., 2003). 812 patients with moderate to severe COPD and mean FEV1 36% predicted were recruited. The combination therapy significantly reduced the number of severe exacerbations (defined as those that needed oral steroids and/or antibiotics and /or hospitalisation) per patient per year compared to placebo and formoterol. Combination therapy also reduced mild exacerbations (defined as use of short-acting bronchodilators for one day more than the usual daily needs) compared to placebo. The combination

therapy also increased FEV1 significantly compared to placebo and budesonide. It also significantly increased and maintained morning and evening PEF compared to the other groups, significantly reduced symptoms within the first week of treatment, reduced the use of short-acting bronchodilators compared to placebo and budesonide and improved quality of life compared to placebo. Budesonide or formoterol alone also significantly reduced mild exacerbations and increased FEV1 and increased and maintained morning and evening PEF compared to placebo. There were significantly fewer withdrawals in the active treatment groups compared to placebo.

Budesonide combined with formoterol (200/4.5 µg twice daily) was compared to placebo and each individual compound (200 µg budesonide or 4.5 µg formoterol twice daily) in another study (TRial of Inhaled Steroids ANd long-acting β₂ agonists, TRISTAN) (P. M. Calverley et al., 2003). 1022 participants with severe and very severe COPD (mean±SD FEV1% predicted 36±10, BDR ~ 6 ±6%) were recruited in the study. The study had a 2 weeks run-in period during which all patients were treated with oral prednisolone (30 mg) and formoterol (4.5 µg twice daily), then they were divided into study groups without oral prednisolone. Withdrawal from the study occurred less frequently with combined therapy than the other drugs. Compared to other treatments, combined therapy significantly prolonged the time to first exacerbation and increased FEV1. This group had also fewer exacerbations per year compared to placebo and formoterol, and significant reduction of SGRQ score [lower score indicates improvement (Carolyn B. Wilson, Jones, O'Leary, Cole, & Wilson, 1997)] compared to all other treatments. Formoterol also significantly improved FEV1 compared to placebo. FEV1 stayed higher after the run-in period with combined therapy during the study time but not with the other treatments, i.e. FEV1 declined rapidly in the other groups after oral prednisolone cessation. Morning PEF was significantly higher with combination therapy compared to placebo and each individual treatment, but evening PEF was higher only compared to placebo and budesonide. This improvement in PEF has maintained throughout the study. Budesonide or formoterol significantly reduced SGRQ score compared to placebo.

The effects of combination of ICS plus LABA were compared with placebo by Cochrane Systematic Review (L. Nannini et al., 2004). Six randomised, double-blind trials with 4118 participants were included. Combination therapy was more effective than placebo in reducing rate of exacerbations, improving quality of life and lung function.

Cochrane Systematic Review collated published investigations which compared ICS combined with LABA with LABA alone (L. J. Nannini et al., 2007b). Ten double-blind randomised trials with 7598 participants with severe COPD were included. Eight studies evaluated FP plus salmeterol combination and two studies budesonide plus formoterol combination. Combination therapy reduced the rate of exacerbations, improved quality of life, and resulted in better FEV1 compared to LABA alone. The study did not find a significant difference in mortality rate and rate of hospitalisation between two methods of treatment. Pneumonia occurred more frequently in the combination treatment group. The authors concluded that combination therapy is more effective than LABA alone to reduce exacerbation rates.

ICS was also compared to combination treatment with ICS plus LABA by Cochrane Systematic Review (L. J. Nannini et al., 2007a). Seven randomised controlled trials with 5708 participants were included. Combination therapy significantly reduced exacerbation rates, including exacerbations that needed oral steroids. The subjects on combination therapy also had reduced mortality rate, better quality of life and, better lung function and fewer withdrawals compared to ICS alone. Side effects were not different.

An international, double blind, randomised study conducted in 20 countries recruited 1323 patients with COPD (GOLD III and IV, mean post-bronchodilator FEV1% predicted was ~ 39% in both groups) (Wedzicha et al., 2008). The study compared salmeterol plus FP combination (SFC, 50/500 µg/twice daily) with tiotropium 18 µg/daily [tiotropium is a long-acting anticholinergic which is used as a dry powder inhaler by a device called Handihaler]. The treatment period was two years. Withdrawal

from the study was significantly greater in the tiotropium group. Mortality was significantly lower with SFC. SGRQ score was significantly lower in the SFC group [lower scores indicate better quality of life (Carolyn B. Wilson et al., 1997)]. The rate of exacerbations was not different between the two treatment groups.

A multi-centre, randomised, double blind controlled study which recruited 449 moderate or severe COPD patients in Canada compared tiotropium plus placebo with tiotropium plus salmeterol or with tiotropium plus fluticasone-salmeterol combination (Aaron et al., 2007). The treatment period lasted one year. The primary endpoint, frequency of exacerbations, was not different between groups. Tiotropium plus fluticasone-salmeterol compared to tiotropium plus placebo had greater effects on lung function (indicated by increase in FEV1 and FEV1% predicted), disease specific quality of life (measured with SGRQ), reduced the frequency of hospitalisations for acute exacerbations of COPD and also all-cause hospitalisations. Tiotropium plus salmeterol also improved quality of life compared to tiotropium plus placebo. The authors noted that more than 40% of subjects on tiotropium plus placebo and tiotropium plus salmeterol withdrew from the study and many patients used open labeled ICS or LABA during the study, which was analysed as intention to treat.

A randomised, double blind three-way cross-over study compared triple therapy (tiotropium 18 µg plus salmeterol and FP 50/500 µg twice daily) to tiotropium alone or SFC in 41 COPD patients for 13 weeks (Singh et al., 2008). It found that the triple therapy significantly improved specific airway conductance better than tiotropium or SFC alone on day 14. [Airway conductance (Gaw) is the reverse of airway resistance (Raw) and is calculated as flow (liters per unit of time) divided by pressure gradient (alveolar pressure – mouth pressure in mmHg)]. Inspiratory capacity (IC) also reduced significantly in the triple therapy group better than the individual- therapy groups. The triple therapy group had significant improvement in dyspnea score and the need for rescue medications. [Rescue medications in COPD are used for immediate relief of dyspnea attacks and include short-acting beta adrenergic agonists or short-acting anticholinergics].

Summary: This wealth of clinical trial data shows that ICS undoubtedly have positive effects in COPD, even when given alone, but especially when given combined with LABA.

13.c.2-Antiinflammatory effects:

A few studies have evaluated the antiinflammatory effects of steroids in COPD (Table 2-2).

Reid et al. compared FP 500 µg twice daily for 6 months (number = 19) with placebo (number = 11) in a randomised double blind clinical trial in mild to moderate COPD. They found that FP improved FEV1, FEV1% predicted and BDR significantly greater than placebo. In within-groups analysis, BDR also improved significantly in the FP group but not in the placebo group and FEV1 increased with FP but did not reach statistical significance ($p = 0.07$). FP improved clinical manifestations significantly including St George's Respiratory Questionnaire (SGRQ) score and breathlessness. Activity was also improved with FP but missed significance narrowly ($p = 0.06$). The placebo group also had a significant improvement in shortness of breath. There was no difference between the groups for improvement in clinical manifestations (D. W. Reid et al., 2008). The study did not find significant changes in sputum inflammatory cells after treatment in either group. BAL neutrophils significantly reduced in the active treatment group, but not in the placebo group. Between-group difference for BAL neutrophils was also significant. In BB, neutrophils increased with FP but macrophages, CD8+ cells and mast cells decreased. There were significant between-group differences for neutrophils and macrophages. The authors discussed that sputum was a poor predictor for monitoring inflammatory cell changes in the airway walls (D. W. Reid et al., 2008). The subjects consisted of current and ex-smokers. Subjects with history of asthma were excluded from the study but BDR, defined according to the ATS and BTS guidelines (Miller et al., 2005; Siafakas et al., 1995) was not an exclusion criteria; 10 subjects had significant BDR according to ATS guidelines. The changes in the airway wall were

Table 2-2. Summary of published studies on response of inflammation to ICS in COPD*

Study group	Drugs	Methods	Main results
(D. W. Reid et al., 2008)	FP 500 µg /bd x 6 months vs. Placebo	RCT Mild to moderate COPD 30 subjects 10 subjects with BDR	Within-group analyses: FP reduced BAL neutrophils. Reduced macrophages, CD8+ and mast cells in BB. Between-groups analyses: FP reduced BAL neutrophils. Reduced macrophages in BB.
(N. C. Barnes et al., 2006)	SFC 50/500 µg/bd x 13 weeks vs. Placebo	RCT 140 subjects Moderate to severe COPD No significant BDR	SFC vs. placebo: Lung function improved. Sputum neutrophil differential reduced. Sputum eosinophils reduced. CD8+ cells, CD4+ cells and CD45+ cells in BB reduced. Reduction in CD8+ in BB.
(Culpitt et al., 1999)	FP 500 µg/bd x 4 weeks vs. Placebo	RCT, cross-over 13 subjects Moderate to severe COPD	FP vs. placebo No change in symptoms or lung function. No change in sputum neutrophils, lymphocytes, macrophages and eosinophils. No change in sputum IL-8, MMP-2, MMP-9 or TIMP.

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Table 2-2. Summary of published studies on response of inflammation to ICS in COPD* (continued)

Study group	Drugs	Methods	Main results
(Hattotuwa et al., 2002)	FP 500 µg/bd x 3 months vs. placebo	RCT 30 subjects All but 4 had moderate to severe COPD No significant BDR	FP vs. placebo: Symptoms improved. No change in lung function. No change in neutrophils, CD8+ cells and CD68+ cells in BB. Reduced CD8:CD4 ratio in the epithelium and mast cells in the LP.
(Gizycki, Hattotuwa, Barnes, & Jeffery, 2002)	Same as Hattotuwa et al.	Used electron transmission microscope for 24 subjects from the same sample as Hattotuwa et al.	FP vs. placebo: Mast cells reduced in BB both within-group between FP and placebo groups.
(Keatings, Jatakanon, Worsdell, & Barnes, 1997)	Budesonide 800 µg/bd x 2 weeks followed by prednisolone 30 mg/d x 2 weeks	No placebo control 13 subjects Severe COPD	No change with either treatment in symptoms and lung function. No change in sputum inflammatory cells, TNF-α, eosinophil activation or neutrophil activation
(T. r. s. S. Lapperre et al., 2009) (GLUCOLD)	FP 500 µg/bd for 6 months then either continued up to 30 months or replaced by placebo up to 30 months, or: SFC 50/500 µg/bd for 30 months, or: Placebo 30 months	RCT 114 moderate to severe COPD current-/ex-smokers For 6 months and 30 months BB and sputum were examined	FP vs. placebo for 6 months: Reduced CD3+, CD4+, CD8+ and mast cells in BB, effects maintained on FP after 30 months. FP for 30 months vs. placebo: Reduced mast cell count in BB. Reduced sputum neutrophils, lymphocytes and macrophages. Improved FEV1 decline, dyspnea and QOL. Improvement in clinical and inflammatory parameters correlated. Withdrawal of FP worsened inflammation. Adding salmeterol improved FEV1.

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Table 2-2. Summary of published studies on response of inflammation to ICS in COPD* (continued)

Study group	Drugs	Methods	Main results
{ Bourbeau, 2007 #298	SFC 50/500 µg twice daily or: FP 500 µg twice daily vs. Placebo All for three months	RCT 60 COPD subjects, stage I-IV	SFC vs. placebo: Reduced CD8+ cells and CD68+ cells.
(Ozol et al., 2005)	Budesonide 400µg twice/bd x 6 months vs. Placebo	RCT 26 subjects with mild to moderate COPD BDR<12% and <200 ml	Budesonide vs. placebo: Reduced BAL IL-8 and mean percentage of neutrophils.
(Confalonieri et al., 1998)	Beclomethasone 500 µg three times daily x 2 months vs. No medication (control)	Randomised open study 34 COPD subjects current smokers	Total cells and neutrophils reduced in sputum compared to baseline in the treatment group, but not controls, and also with treatment compared to control.
(Boorsma et al., 2008)	Budesonide 800 µg/daily x 6 months vs. Placebo	RCT 19 subjects with COPD mean FEV1% predicted 65%	No change in sputum neutrophilia. Sputum eosinophil% reduced with budesonide at 3 months but not at 6 months.

* bd = Twice daily, BD = bronchodilator, BDR = Bronchodilator responsiveness, FP = Fluticasone propionate, Hx = history, PBD = post-bronchodilator, RCT = Randomised clinical trial, SFC = Salmeterol plus fluticasone combination

similar to those other have shown with ICS/LABA combination (see below) apart from the increase in wall neutrophils, which is likely to be lack of neutrophil clearance, which LABA might overcome.

Antiinflammatory effects of salmeterol plus FP combination (SFC, 50/500 µg twice daily) were evaluated in a 13-week randomised placebo controlled study in 140 moderate to severe COPD patients who did not have significant BDR (N. C. Barnes et al., 2006). After treatment sputa were analysed for neutrophil numbers at 8 and 13 weeks. BB were compared between groups for CD8+ and CD68+ cells after 12 weeks. SFC reduced CD8+ cells significantly. Also, compared to placebo the change in CD8+ cells was significantly greater in the SFC group. Sputum neutrophil differential (not total number) reduced significantly in the SFC group after 13 weeks of treatment. SFC also significantly reduced total sputum eosinophils, biopsy CD4+ cells and biopsy CD45+ cells. There was also a significant improvement in lung function.

A double blind, placebo controlled, cross over study used FP 500 µg twice daily in 13 moderate to severe COPD subjects (BDR<15%, 11 current smokers) for 4 weeks. Sputum only was examined for inflammatory cells (neutrophils, lymphocytes, macrophages and eosinophils), IL-8, MMP-2, MMP-9 and TIMP. The study did not find any change in symptoms, lung function parameters and sputum cells and mediators after treatment. The authors concluded that inflammation and protease activity in COPD are resistant to high dose ICS (Culpitt et al., 1999) but, as above, sputum seems to lack sensitivity to the effects of ICS (D. W. Reid et al., 2008). Culpitt et al. admitted that their study might not have been sufficiently long, but cited another study that did not find any improvement in lung function with ICS over three months (A. Watson, Lim, Joyce, & Pride, 1992). However, Watson et al. mainly recruited mild to moderate COPD subjects and their primary outcome was to measure changes in bronchial hyper-responsiveness (BHR).

Another study from the Barnes group reported no significant difference in neutrophils, CD8+ and CD68+ cells in BB from 30 COPD subjects after treatment with FP (number

= 16) 500 µg twice daily or placebo (number = 14) for three months, but reported a significant reduction of CD8:CD4 ratio in the epithelium and number of mast cells in the LP (Hattotuwa et al., 2002). Within-group analysis showed significant decrease in mast cell number in the FP group. Correction for multiple analyses (although not technically appropriate) resulted in loss of all significant differences except for mast cell changes in the FP group that still remained close to statistical significance ($p = 0.06$). Cough and sputum, and the need for short-acting bronchodilators showed significant improvements in the FP group, but there were no significant changes in lung function parameters. Participants did not have significant reversibility with bronchodilators and mainly suffered from moderate to severe COPD, except for 4 subjects that had mild COPD. This group used the same samples for electron transmission microscopic examination to evaluate the results of treatment with FP (number = 14) or placebo (number = 10) subjects (Gizycki et al., 2002). The placebo group experienced significantly more exacerbations compared to the FP group during the trial. Compared to the baseline and to placebo, FP resulted in a significant reduction in mucosal mast cells. Mucosal neutrophils increased in the active treatment group. Mononuclear cells did not change. The authors proposed that the selective anti-inflammatory effect of ICS to reduce mast cells might be related to the improvement in clinical manifestations.

Another group that had previously reported the increase in neutrophils and inflammatory mediators (IL-8 and TNF- α) in the sputum of COPD subjects (Keatings et al., 1996), investigated the effects of ICS (budesonide 800 µg twice daily) followed by oral steroid (prednisolone 30 mg daily) for 2 weeks on these inflammatory markers (Keatings et al., 1997). Neither form of steroids effectively improved lung function and inflammatory sputum parameters.

Effects of 6 months and 30 months treatment with FP 500 µg/twice daily alone or combined with salmeterol (SFC) 50/500 µg/twice daily were compared with placebo in a randomised clinical trial (Groningen Leiden Universities Corticosteroids in Obstructive Lung Diseases, GLUCOLD), on 114 moderate to severe COPD subjects (T. r. s. S. Lapperre et al., 2009). Patients were randomised in four groups: FP for 6 months and

then placebo up to 30 months, FP for 30 months, SFC for 30 months and placebo for 30 months. CD3+, CD4+, CD8+ and mast cells reduced in BB after 6 months of treatment with FP compared to placebo; these effects persisted after 30 months and mast cells reduced further in those that continued FP. After 30 months of FP, sputum neutrophils, macrophages and lymphocytes reduced and dyspnea, quality of life and lung function parameters improved compared with placebo. Withdrawal of FP at 6 months worsened inflammation, i.e. CD3+ cells, mast cells and plasma cells increased in BB, but sputum cells did not change significantly. Withdrawal also intensified FEV1 decline and worsened clinical manifestations. Adding salmeterol to the treatment improved FEV1. At 6 months, SFC treatment resulted in better FEV1 and dyspnea score than FP alone. FEV1 improvement correlated with decrease in CD4+ cells.

Bourbeau et al. compared SFC 50/500 µg/twice daily (n = 19) or FP 500 µg/twice daily (n = 20) with placebo (n = 21) in a randomised controlled trial. BB were compared before and after 3 months of treatment. Patients with mild to very severe disease were included; patients with FEV1% predicted <25% were excluded. SFC, but not FP reduced CD8+ cells and CD68+ macrophages compared to placebo (Bourbeau et al., 2007). Neutrophils and eosinophils did not change significantly with treatment when compared to placebo.

The effect of Budesonide 400 µg/twice daily was compared with placebo in a randomised controlled trial to evaluate inflammatory markers in BAL in 26 subjects with mild to moderate COPD. Budesonide reduced IL-8 and percentage of neutrophils in BAL (Ozol et al., 2005). Subjects had a BDR <12% and <200 ml.

Beclomethasone dipropionate 500 µg three times daily for 2 months was compared with no medication (control group) in a study to assess the effects of treatment on inflammatory cells in sputum of 34 COPD patients (Confalonieri et al., 1998). The study showed total cell and neutrophil reduction compared to baseline in the treatment group but not in control group. The differences between treatment and control groups were

significant at the end of the study for neutrophils and total cells. Macrophages increased significantly in the treatment group compared to the control group.

Boorsma et al. evaluated the effect of Budesonide 800 µg daily for 6 months in a double blind, randomised, placebo controlled cross-over clinical trial. 19 COPD subjects (mean FEV1% predicted 65%) were recruited. There was no change in sputum neutrophils with either treatment. Budesonide reduced sputum eosinophil% compared to placebo at 3 months but not at 6 months (Boorsma et al., 2008).

Summary: The data available shows fairly consistent positive effects of ICS on cellular inflammation, especially in the airway wall. None of the specific changes in themselves seems related to the corresponding improvements in symptoms and lung function. What seems definite is that the airway inflammation in COPD is not ICS-unresponsive, although it may well be that ICS do not affect the most pathophysiological important changes, but as yet we do not know what these are.

13.c.3-Effects on airway remodelling:

To my knowledge, there are no published longitudinal studies addressing the effects of ICS on airway remodelling in COPD.

A recent cross-sectional study compared BB from COPD subjects who were on ICS (beclomethasone dipropionate) 1.6-2.4 mg daily (number = 10), COPD subjects who were not on ICS (number = 10) and a control group (number = 8) (Zanini et al., 2009). The controls underwent diagnostic bronchoscopy for haemoptysis or peripheral lung nodules. Subjects with COPD had quit for at least 10 years but had clinical criteria for chronic bronchitis and suffered from stable moderate to severe airflow obstruction. Vascular area, vessel size, VEGF positive cells and TGF-β positive cells were significantly increased in the LP of the COPD group that did not take ICS compared to the ICS and placebo groups. The number of vessels was not different between the groups. The COPD group had also significantly more CD8+ and CD68+ cells. Vascular area had positive correlations with cells positive for VEGF and cells positive for TGF-β.

Thus, the authors proposed that vascular changes in COPD might respond to ICS. However, with a cross-sectional study, it is hard to make this conclusion very firmly. Furthermore, the COPD group was not very typical.

Although not really related to airway remodelling, it is worth citing a double-blind randomised study that compared an ICS (budesonide 400 µg twice daily) with placebo for 2-4 years to evaluate the effects of corticosteroids in the progression of *emphysema*. It did not find significant differences between groups but there was a trend towards a protective effect of budesonide, i.e. annual decline of lung density was less than placebo, but there was no difference in the rate of decline of FEV1 between the two groups (Shaker et al., 2009).

Overall summary: Multiple reports confirm limited clinical benefit for using inhaled corticosteroids alone or in combination with LABA for patients with moderate to severe COPD. Strong evidence supports they improve symptoms, reduce the frequency and severity of acute exacerbations of disease, improve lung function and affect inflammatory changes in airway tissue.

The benefits of ICS in more severe COPD seem well established, but using ICS in “milder” than stage III and IV COPD, considering its potential adverse effects in the elderly, seem much less certain. Investigators of a large, multi-center studies support this conclusion (TLHSRG, 2000). Calverley et al.’s study separately analysed COPD subjects with severe disease and those with less severe disease and suggested that ICS were more effective in subjects with FEV1<50% predicted (P. Calverley et al., 2003). Reports on antiinflammatory effects of ICS on sputum and biopsies suggest that sputum samples are not as good as asthma in monitoring inflammation in stable COPD. However, reports on biopsy samples from COPD subjects support that ICS have antiinflammatory effects on this disease.

13.d-Steroid “resistance”:

As said before, therapeutic effects of steroids are not as marked in stable COPD as they are in patients with asthma. This is referred as steroid “resistance” in quite a few papers. However, it should be kept in mind that it is probably not reasonable to expect the same effect from steroids in stable COPD as in asthma. As discussed before, these two diseases are quite different in many aspects (see Section 12, COPD versus asthma). Besides, the literature provides very good support for the efficacy of ICS in patients with COPD (see above, Section 13.c, Use in COPD). For these reasons, I would not consider COPD as resistant to but only relatively insensitive to steroids.

There are multiple potential mechanisms proposed for the relative corticosteroid resistance in inflammatory diseases including COPD and indeed resistant asthma (P. J. Barnes & Adcock, 2009; Rhen & Cidlowski, 2005):

- Genetic resistance, e.g. polymorphisms in the steroid receptor gene or associated chaperone proteins
- Defective nuclear transport of GR
- Increased expression of glucocorticoid receptor β : this receptor competes with glucocorticoid receptor α for binding to glucocorticoid response elements and thus, has an inhibitory effect on the function of glucocorticoids
- Excessive activation of activator protein 1 (AP1): AP1 protein binds to the glucocorticoid receptor and blocks its interaction with glucocorticoid response elements
- Abnormal histone acetylation: Steroids are not able to switch on glucocorticoid-responsive genes, such as mitogen-activated protein kinase phosphatase 1, via acetylation of lysine residues on histone 4. This enzyme is an inhibitor of mitogen-activated kinase (MAP kinase) pathway which produces several inhibitory mediators.
- HDAC2 inactivation
- Increased activity of multi-drug resistant gene: MDR1, this gene encodes p-glycoprotein-170 which can pump glucocorticoids out of cells
- Increased macrophage migration inhibitory factor (MIF), which has anti-glucocorticoid effects

- Phosphorylation of GR by MAPK

None of these are definite. However, the main mechanism for relative resistance to steroids in COPD is suggested to be intense reduction of histone deacetylase-2 (HDAC2) expression in airways and peripheral lung (P. J. Barnes & Adcock, 2009). Acetylation of histone by histone acetyl transferase (HAT) unwinds chromatin and therefore transcription factors are able to have access to DNA and a pro-inflammatory transcription complex is produced (Figure 2-19, insets a & b). HDAC is a phosphoprotein and is a repressor of production of pro-inflammatory cytokines by reversing histone acetylation (P. J. Barnes, 2006). Interestingly, this enzyme is adequately present in asthma (Kazuhiro Ito et al., 2005; To et al., 2010). As said before, oxidative stress activates PI3K- δ resulting in the activation of downstream kinases such as AKT (protein kinase B) which cause phosphorylation and inactivation of HDAC2 (Figure 2-23) (P. J. Barnes & Adcock, 2009) and thus impair the ability of the GR-steroid complex to access its transcription sites. Peroxynitrite, formed by oxidative and nitrative stress, nitrates the tyrosine residues of HDAC2 also resulting in its inactivation. HDAC2 activity reduction also causes direct hyperacetylation of glucocorticoid receptors and this also inhibits corticosteroids getting access to their DNA binding sites to switch off inflammatory genes (P. J. Barnes, 2009; To et al., 2010).

In a study that was carried out on both human and mice, To et al. tested the mechanisms of corticosteroid resistance in COPD. They compared dexamethasone (a corticosteroid) sensitivity in COPD subjects with nonsmoking and smoking controls. Sensitivity was tested as the 50% inhibitory concentration of dexamethasone on TNF- α -induced IL-8 release in peripheral blood mononuclear cells. COPD subjects were significantly less sensitive than the controls. Corticosteroid insensitivity was reversed with nonselective inhibitors of PI3K- δ enzyme and low dose theophylline. Theophylline also blocked oxidant-induced activation of PI3K- δ . In cigarette smoke exposed mice, selective PI3K- δ and theophylline reversed dexamethasone insensitivity. The authors concluded that

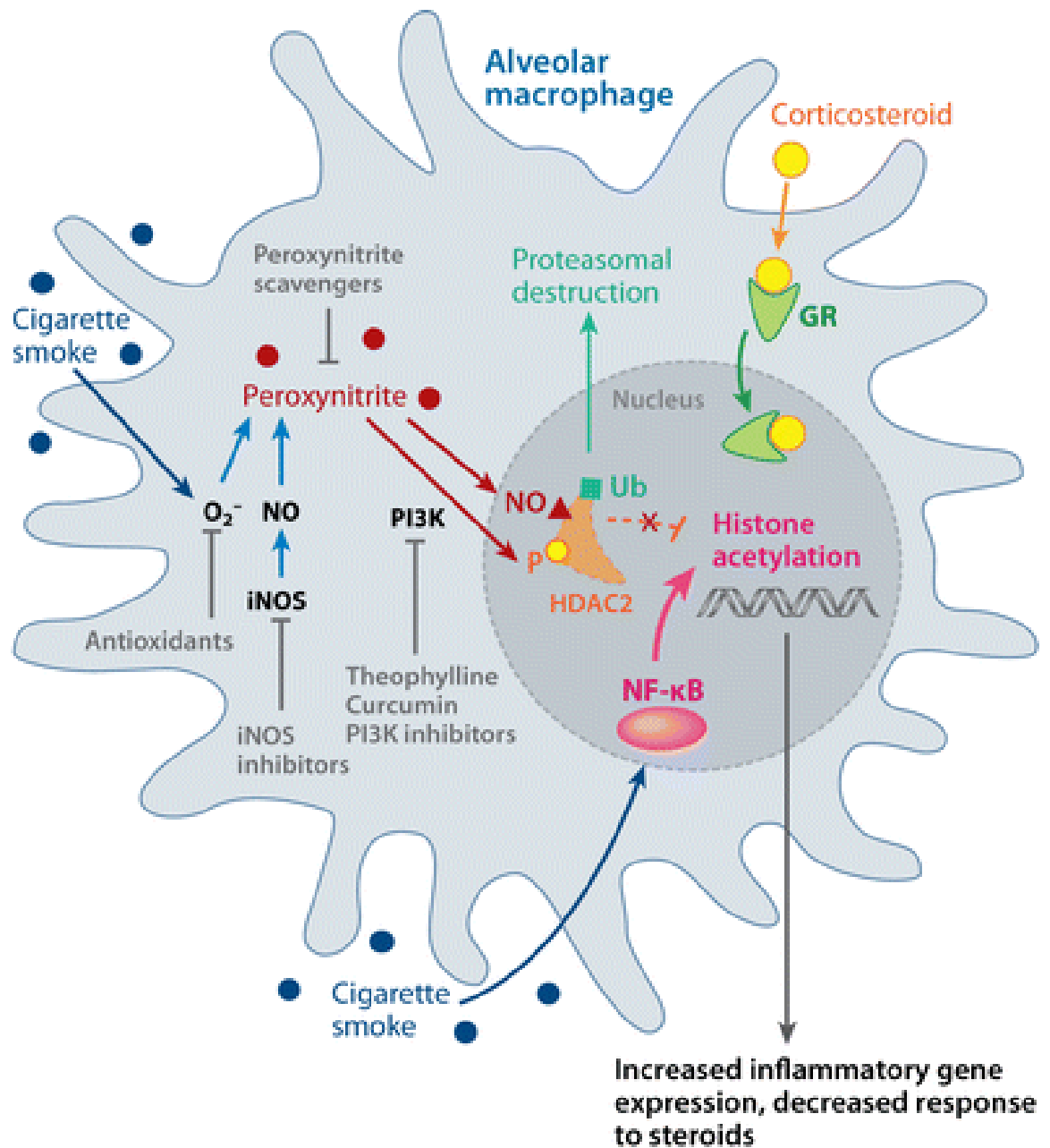


Figure 2-23. Possible mechanisms of “resistance” to ICS in COPD. Peroxynitrite, a product of superoxide anions (O_2^-) and nitric oxide (NO), degrades HDAC2. This may be followed by ubiquitination (Ub) of the enzyme and degradation by the proteasome. PI3K enzymes which are activated by oxidative stress inactivate HDAC2. Inactivation of HDAC2 then enhances inflammatory genes transcription and loss of ICS function. (source: (P. J. Barnes, 2009)).

inhibition of oxidative induced activation of PI3K- δ is a novel approach to reverse corticosteroid insensitivity in COPD (To et al., 2010). The major question which a broad look at the data raises, however, is the extent to which COPD in humans can really be regarded as ICS-resistant and if it is, what are the strategic resistant mechanisms.

Chapter Three

Material and Methods (General Methodology)

To test my hypotheses (see chapter one) I designed both *cross-sectional* and *longitudinal analyses* to examine the characteristics of remodelling, especially vascular remodelling, in the airways of smokers and COPD subjects and to assess the effects of inhaled corticosteroids (ICS) on these changes. For both studies, I used the tissues that remained from the bronchial biopsies that were taken for a previous PhD student (Yudong Wen). My comprehensive cross-sectional study was designed on the basis of the information gained from two sequential *pilot studies*. These preliminary pilot studies guided us to focus on Rbm and vascular remodelling in the LP of the mucosa for my comprehensive study. Thereafter I tested the effects of ICS on these components of remodelling. I also compared our biopsies stained for Factor VIII and Collagen IV to investigate whether these two techniques of vessel staining gave the same or different signals.

Ethics committee approval: All studies were approved by the Joint Human Research Ethics Committee (Tasmania).

Cross-sectional study:

The cross-sectional study was designed to define the characteristics of large airway vascular remodelling in COPD in detail and to determine if these characteristics are specific for COPD or not.

Subjects: Subjects were recruited by advertisement. All studies were carried out in Tasmania, Australia. To test our hypotheses we used bronchial biopsies (BB) from current smoker COPD (S-COPD), ex-smoker COPD (ES-COPD) as well as a healthy nonsmoker control group (H-N) for our cross-sectional study. We also recruited subjects who smoked and had normal lung function (S-N) to test if our potential findings were a consequence of exposure to smoke or were actually related to COPD itself as a disease entity.

Our inclusion criteria for COPD subjects were:

- Age > 40 years old
- History of smoking equal to or more than 15 pack-years. (Pack-year: Number of packs of cigarettes smoked on average day x number of years of smoking. For example if somebody has smoked 1 pack of 20 cigarettes everyday for 10 years, he has a 10 pack-year smoking history)
- Post-bronchodilator FEV1/FVC ratio equal or < 69%
- In order to cope with bronchoscopy, all subjects were in GOLD stages I or II (GOLD, 2007), i.e. FEV1 50% predicted or greater

Exclusion criteria were:

- History of asthma and other respiratory diseases except COPD
- Use of corticosteroids over last 12 weeks
- Use of long acting β -adrenergics over 4 weeks
- History of exacerbation or symptoms of bronchitis over last 4 weeks
- History of any significant and uncontrolled co-morbidities
- Saturation of arterial oxygen equal or less than 92% at rest
- Contraindications for FOB

- Inability to give informed consent

Inclusion criteria for S-N:

- Age of at least 18 years
- Currently smoking and history of continuous smoking for at least 5 pack-years
- FEV1/FVC ratio of 70% or higher and FEV1% predicted of 80% or greater

Exclusion criteria for S-N:

- History of asthma or other respiratory diseases
- History of any significant and uncontrolled co-morbidities
- Inability to give informed consent

Inclusion criteria for H-N:

- Age of at least 18 years
- Being life-long nonsmoker
- No history of asthma or other respiratory diseases
- FEV1/FVC ratio of 70% or greater and FEV1% predicted of 80% or greater

Exclusion criteria for H-N:

- Same as for S-N

Longitudinal study:

The longitudinal study was a randomized double-blind placebo controlled clinical trial. The aim of this study was to compare the effects of fluticasone propionate (FP) with placebo in airway remodelling in BB from COPD participants.

Subjects: The COPD subjects who volunteered for our cross-sectional study were asked to participate in the clinical trial.

Inclusion and exclusion criteria were as previously mentioned for COPD participants in the cross-sectional study.

Study design is shown in Figure 6-1 in Chapter Six.

Lung function tests:

Spirometric and lung volume measurements were performed using Sensor Medics (CA, USA) spirometer and body box. FVC and FEV1 were measured according to the American Thoracic Society (ATS) guidelines (Miller et al., 2005). The best values of three technically correct measurements were selected. The values were reported as body temperature and pressure water saturated (BTPS).

Vital capacity (VC) is the volume that someone exhales completely out after a deep inhalation, i.e. from total lung capacity (TLC) to residual volume (RV) (Figure 3-1). When VC is measured during a forced exhalation (Figure 3-2) it is called forced vital capacity (FVC). During FVC maneuver, the volume exhaled in the first second is called forced expiratory volume in one second (FEV1). Normal subjects can expel 70% or greater of their FVC in one second; in other words in normal conditions FEV1/FVC ratio x 100 is greater than or equal to 70%. In obstructive airway disease this ratio reduces to 69% or less.

Most modern spirometers can plot the relationship between expiratory or inspiratory flow against lung volume at any time during the FVC maneuver. This makes a loop that is called flow-volume curve (Figure 3-3). Flow-volume curve is a valuable method to diagnose obstructive disease. In obstructive pattern the descending part of the expiratory curve shows scalloping, i.e. maximal expiratory flow rates reduce at any time of forced vital capacity (Figure 3-3B).

Airflow limitation in COPD is the consequence of an increased time constant for inflation and deflation of lung units (Hogg, 2004). When a constant pressure is applied to a lung unit, the time needed to fill a unit is called the time constant which is the product of airway resistance (R_{aw}) and compliance (Ayas, Zakynthinos, Roussos, & Pare, 2010):

$$\text{Time constant} = \text{Raw} \times \text{Compliance}$$

Airway resistance is inversely related to airflow. Compliance has an inverse relationship with elastic recoil pressure, which is a major propelling force to expel air. The less the elastic recoil pressure of a lung unit, the more compliant is the unit (Figure 3-4). In COPD, both airway resistance and compliance of lung units increase (Gold, 2005). The increase in lung compliance is due to emphysema, i.e. breakdown of lung tissue and its elastic properties. These two alterations prolong time constant (Ayas et al., 2010). Therefore, a unit with a longer time constant takes longer to fill and also takes longer to empty. In normal conditions, increasing intrapleural pressure, induced by contracting expiratory muscles, increases expiratory flow rate. But in emphysema this is relatively ineffective, because by increasing positive pressure around airways, small airways also collapse prematurely. When the pleural pressure rises, increased intra-alveolar pressure leads to increase in air flow and intra-airway pressure which tends to act as an internal splint, keeping the airway open. But, this increasing pleural pressure also compresses small airways. These two opposite effects on airway wall cancel each other out if they become equal in the small unsupported airway, which happens when the airway resistance is high as in COPD; consequently increasing expiratory effort has relatively little effect on expiratory flow rate in emphysema.

In COPD FEV1/FVC ratio is 69% or less. FEV1% predicted is calculated by measured FEV1 divided by predicted FEV1 multiplied by 100. The predicted value is calculated by allowing for known age, gender and height. FEV1% predicted is used to determine the severity of airway obstruction (see classification of disease in chapter two). FVC, which represents lung volume, may be normal or reduced in COPD. In COPD, FVC

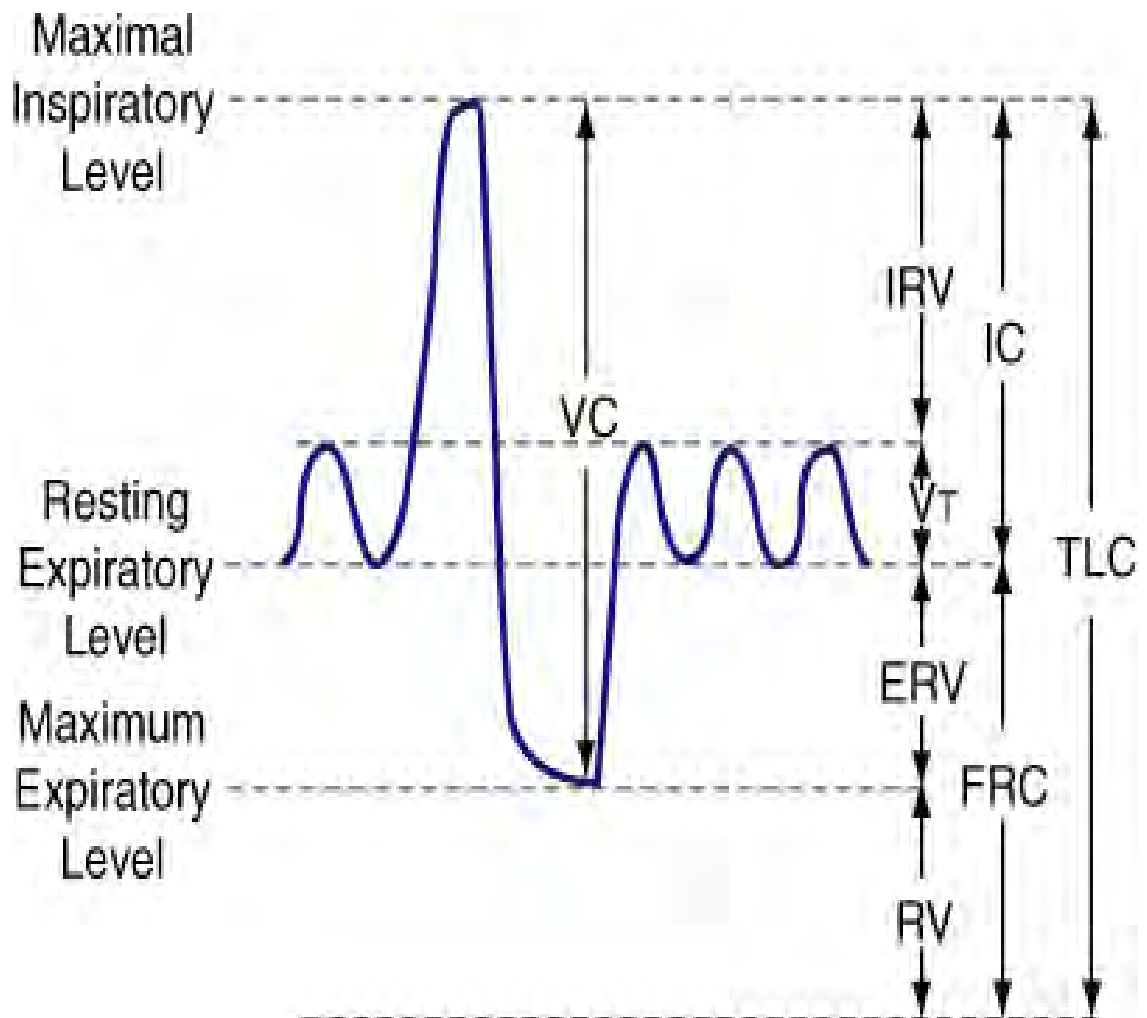


Figure 3-1. Lung volumes and capacities. This is a spirometric diagram. TLC is the total volume of gas in the lungs at the end of a deep and complete inspiration. VC is the volume of gas that one can exhale out from greatest lung volume (TLC) to the residual volume (RV). VC consists of IC (inspiratory capacity) and ERV (expiratory reserve volume). (Source: (Gold, 2005))

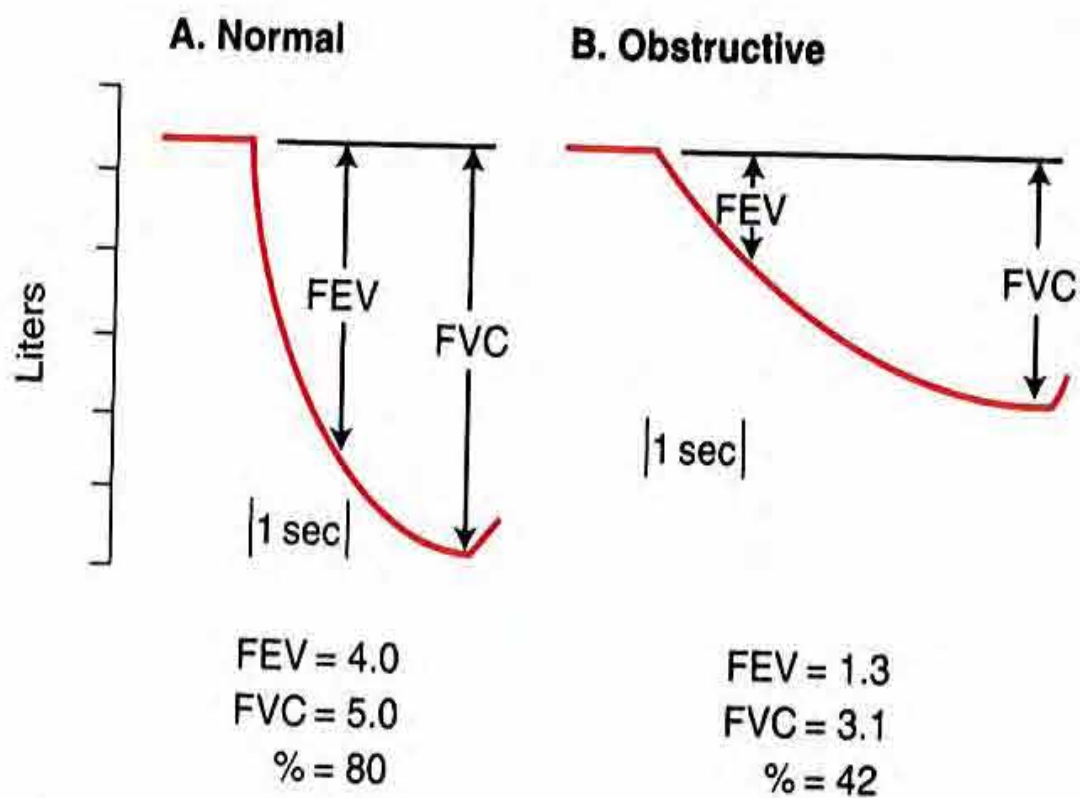


Figure 3-2. *FVC and FEV1 measured by spirometry. An obstructive pattern is compared with normal. (Source: (West, 2003))*

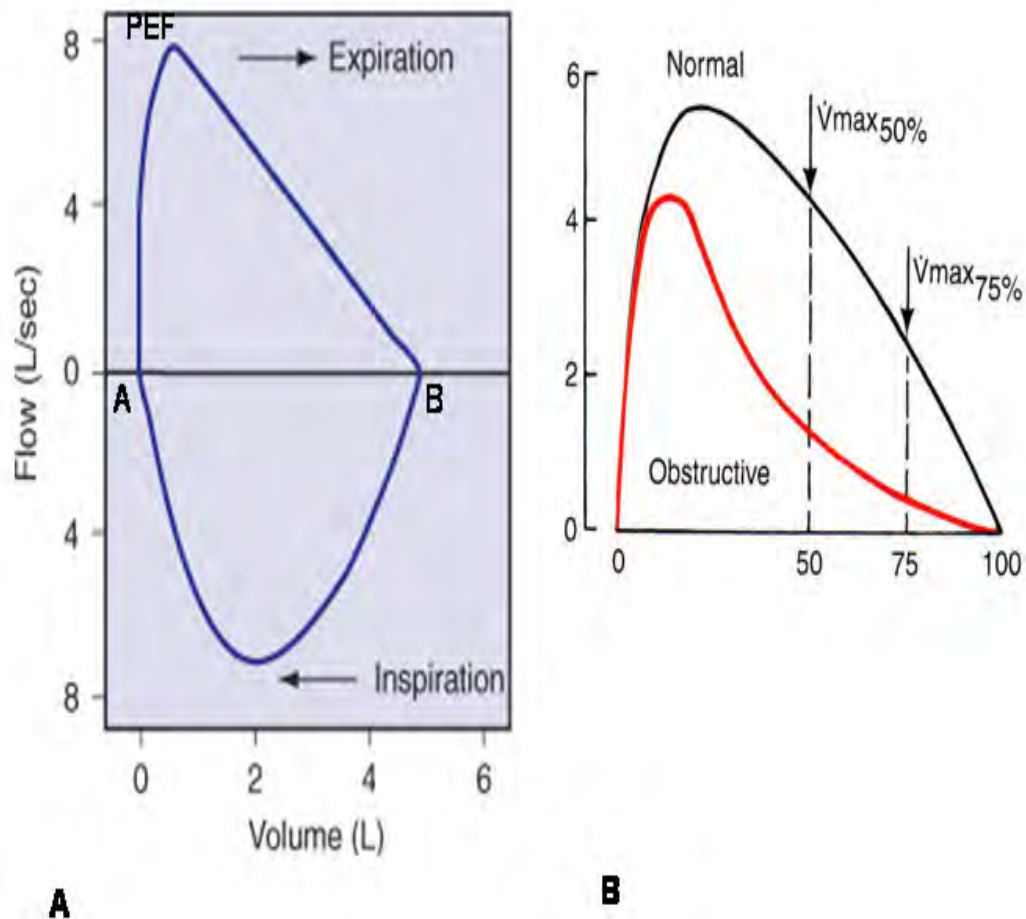


Figure 3-3. A. Flow-volume curve. Expiratory curve is upward and inspiratory curve is downward. A marks lung volume at the point of maximum inflation (TLC) and B marks lung volume at maximal deflation (RV). (Source: (Gold, 2005)) **B.** Flow-volume curve from a patient with obstructive airway disease (inner loop) is compared with normal (outer loop). PEF is reduced and there is scalloping of the expiratory loop, therefore compared to normal loop, maximal flows at 50% and 75% of vital capacity are reduced (Source (West, 2003)). $\dot{V}_{max50\%}$ and $\dot{V}_{max75\%}$ = Maximal expiratory flow in 50% and 75% of FVC, PEF = Peak expiratory flow

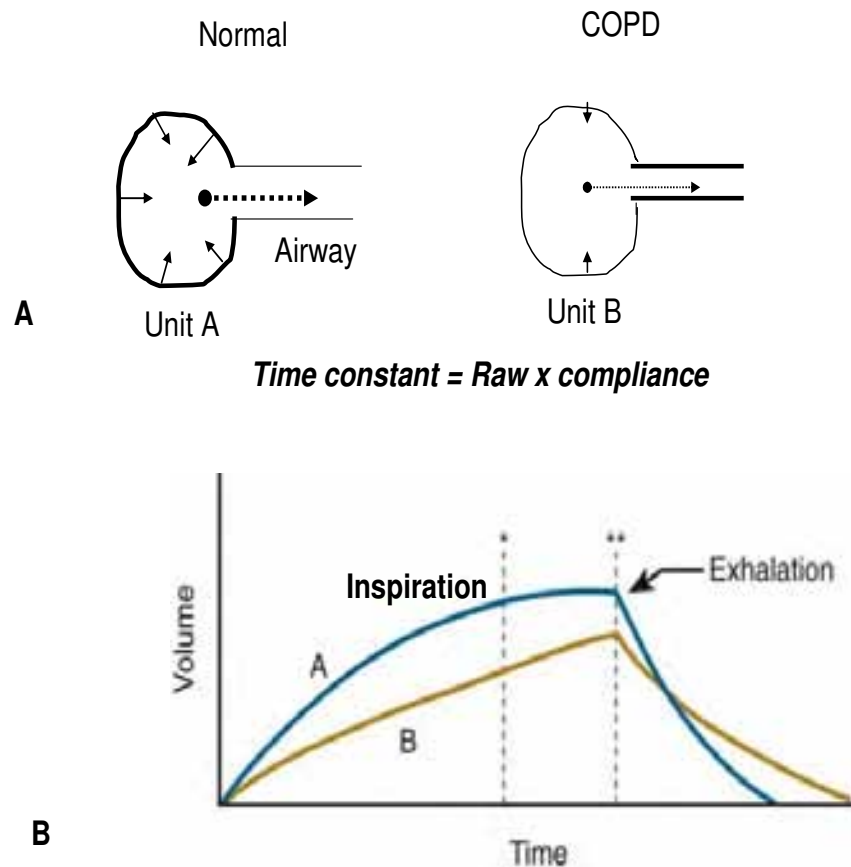


Figure 3-4. A. Upper panel. *Time constant.* The time that takes the lung (balloon) to expel its gas content (dotted arrow) is dependent on the product of compliance of the lung parenchyma (inversely related to elastic recoil pressure, inward arrows) and resistance of the airway (tube). In COPD (unit B) the elastic recoil pressure, which is the major driving force for exhalation, reduces and airway resistance increases (due to airway wall thickening). These two changes prolong the time constant.

B. Lower panel. Two lung units with different time constants are inflated under a constant pressure. Unit A has a shorter time constant, i.e. its airway resistance and/or compliance are less than unit B. Thus it takes a shorter time for it than unit B to be completely filled when a constant pressure is applied (point **). Emptying is also faster in unit A. (Source:(Ayas et al., 2010))

reduction is the result of air trapping. Figure 3-1 shows that there are two mechanisms for FVC reduction, decrease of TLC or increase of RV. RV can increase in COPD as a consequence of bronchiolar involvement causing premature occlusion and so air trapping (GOLD, 2005). Therefore, expiration is not long enough to return to the normal RV. This is called hyperinflation. Patients suffering from COPD have hyperinflated lungs, i.e. they breathe at greater lung volumes than normal subjects. This hyperinflation reduces inspiratory capacity (IC). Total lung capacity (TLC) is also frequently increased in COPD. FEV1 represents the earlier parts of flow exhaled from the lungs. Therefore, it is representative of large airway caliber.

FOB:

Bronchoscopy was performed by A/Professor Richard Wood-Baker and Dr. David Reid as an outpatient procedure as below:

- Subjects stayed fasted overnight.
- Before FOB a post-bronchodilator (200 µg salbutamol inhaler) spirometry was performed.
- Intravenous catheter was inserted.
- Sedation was instituted by intravenous administration of midazolam (5-10 mg) and fentanyl (50-100 microgram).
- Topical lignocaine (200-400 mg) was applied to throat, larynx and airways.
- Olympus Video-bronchoscope (Japan) instrument was used (Figure 3-5). Eight biopsies were taken from the second order subcarina in the right lower lobe with cup forceps.
- Pulse rate, blood pressure and saturation of arterial oxygen was monitored during the procedure.
- There were no complications from the procedure in any of our subjects.

Tissue processing and immunostaining:

All *tissue processing* and *immunostaining* were performed in the Respiratory Research Group immunopathology laboratory in the Menzies Research Institute by a single senior technician/scientist, Mr. Steven Weston.

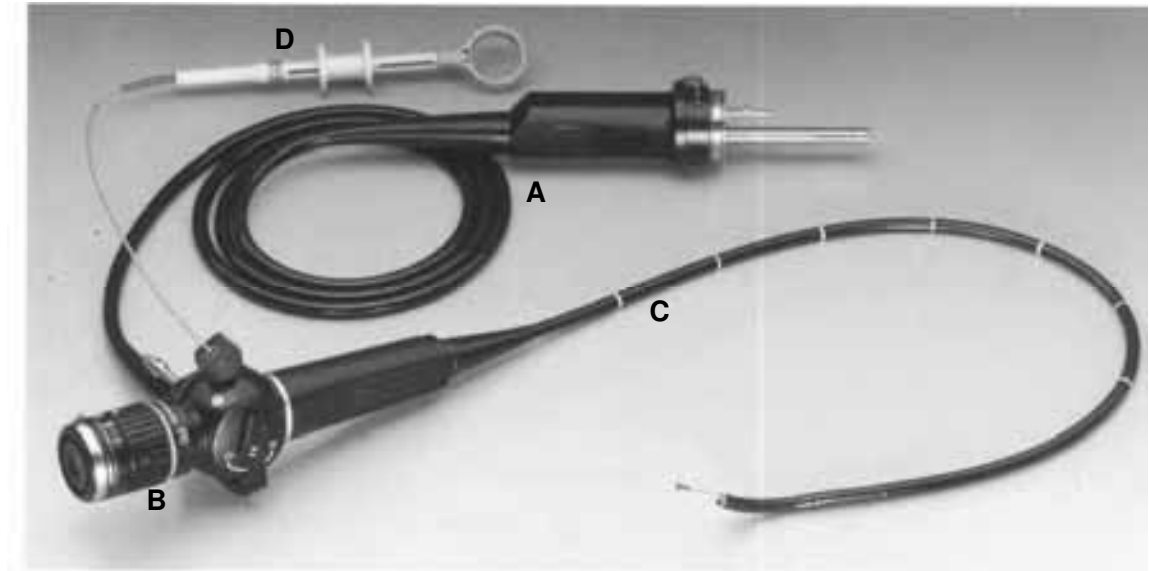


Figure 3-5. *Fiberoptic bronchoscope.* Olympus (Japan) fiberoptic bronchoscope (BF-10) consists of three parts, connection cord to the cold light (A), eye-piece that can be connected to the video scope (B) and flexible shaft (C). The light is transferred from a light source and through the fiber-optics to the shaft which is inserted into the tracheo-bronchial tree through the nose/mouth and the vocal cords during bronchoscopy. A biopsy forceps (D) is inserted through the biopsy canal.

Tissue processing: Four biopsies were collected in saline of which 2 were subsequently snap frozen in liquid nitrogen/isopentane slurry and embedded in OCT for possible immunostaining and the other 2 in liquid nitrogen for molecular analysis at a later date. All 4 were stored at -80°C. The other 4 biopsies were fixed in 4% neutral buffered formalin for 2 hours and subsequently processed into paraffin through graded alcohol and xylene using a Leica ASP 200 tissue processor. Sections were cut at 3 microns from individual paraffin blocks, stained with Haematoxylin and morphologically assessed for immunostaining. Two 3 micron sections from appropriate blocks were collected on each slide being separated by a minimum of 50 microns. Following removal of paraffin and hydration to water, immunostaining for Collagen-IV (Dakocytomation, Denmark, cat. no. M0785 clone CIV 22: 1/100 dilution, 90 minutes at room temp with heat retrieval) and Vascular Endothelial Growth Factor (VEGF) (Fitzgerald, Concord MA. Cat. no. 10R -V101ax: 1/500 dilution, overnight at room temperature) was performed on separate slides. In each case a non immune IgG1 negative control (Dakocytomation, Denmark X0931 clone DAKGO1) was performed to eliminate false positive staining. Bound antibodies were elaborated using Peroxidase labeled Envision + (Dakocytomation, Denmark cat. no. K4001) and liquid DAB + (Dakocytomation, Denmark cat. no. K3468). Sections from all four biopsies were controlled by our laboratory manager and one of them was selected for our measurements on the basis of integrity and the quality of the epithelium and minimal tangential appearance in section cuts.

Immunohistochemical stainings: Tissue staining for different markers followed the instructions below. All tissues were stained with Haematoxylin before immunostaining.

Collagen IV antibody and *Factor VIII* antibody were used to mark blood vessels.

1. Section paraffin blocks at 3 microns after cooling in -20 freezer or on ice blocks for 5-10 minutes.
2. Pick up two sections separated by 40-50 microns (approximately 10 sections) on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides [in distilled water (milli Q water) bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade].

3. Dry 2hrs in slide drying oven.
4. Sort out slides and ensure you have a positive tissue control and a negative reagent control for each section being stained.
5. circle back of slides around sections with xylene resistant pen and put staining date on sections
6. Dewax sections in “xylene” 2 x 5 minutes each in fume hood
7. Hydrate to water using 100% ethanol followed by 95% then 70%, 3 minutes each change
8. Rinse sections well in running tap water (2 minutes)
9. Place in 3% H₂O₂ in distilled water for 15 minutes.
10. Wash in running water 2 minutes
11. Place 500ml of dist water in bottom of pressure cooker
12. Put 500ml of Dako S1700 solution found in door of fridge in plastic incubation vessel (or Dilute concentrate 1 in 10 and reuse)
13. Put slides in S1700 solution and heat in pressure cooker for 6 minutes on high then 14 minutes on about 6 to maintain steam.
14. Allow pressure cooker to cool and then remove lid and place vessel in running water until the solution reaches 35degrees C. Then place sections in running water for 1 minute.
15. Cover slides with PBS pH 7.4 prior to using blocking serum
16. Apply primary antibody to sections for appropriate time at appropriate concentration and temperature. Also apply negative control sera (normal sera from same species as primary antibody) to control section and if possible use reagent control antibody that is well demonstrated in the tissue under investigation.

Add a dilution of 1 in 150 for both Factor VIII (Dako M0616) and Type IV collagen (Dako M0785) (90 minutes at room temperature)

Make up Primary antibody using Dako diluent.

17. Wash sections well using PBS 3 x changes 5 minutes each change
18. Apply DAKO Envision + (Dako k4001 or k4003) reagent to sections for 30 minutes

19. Wash sections well using PBS 3 x changes 5 minutes each change
20. Make up DAKO DAB plus solution (K3468) using 20 micro liters of DAB to 1 ml of substrate buffer (wear gloves)
21. Apply DAB to sections for 10 minutes
22. Wash sections well using PBS for 5 minutes
23. Rinse sections in running water
24. Go down to Anatomical pathology to complete.
25. Place sections in Mayers haematoxylin to elaborate nuclei, 1 min.
26. Rinse in running water
27. Place in approx 400ml of water with 6-8 drops of ammonia for 30 seconds
28. Rinse well in running water
29. Dehydrate in 95% ethanol, and then two changes of 100% ethanol (2 minutes each change) (make sure 95% is not pink, if it is, change it).
30. Clear in three changes of xylene (2 minutes each change) and mount sections in Depex (or similar)
31. Coverslip using machine with 54 mm coverslips.

RDI (Fitzgerald) VEGF staining method:

1. Section paraffin blocks at 3 microns after cooling in -20-degree freezer or on ice blocks 5-10 minutes.
2. Pick up two sections separated by 40-50 microns (approximately 10 sections) on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides [in distilled water (milli Q water) bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. Dry overnight at 37 degrees in incubator.
4. Dewax sections in "xylene" 2 x 5 minutes each in fume hood
5. Hydrate to water using 100% ethanol followed by 95% then 70%, 3 minutes each change
6. Rinse sections well in running tap water (2 minutes)
7. Place in 3% H₂O₂ in distilled water for 15 minutes.
8. Wash in running water 2 minutes

9. Rinse in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 minutes
 10. Block non specific protein using Dako serum free protein block (X0909) 30 minutes
 11. Apply primary antibody (Fitzgerald VEGF Ab-3 cat no RDI-VEGFabm-12 new catalog 10R-V101ax) (clone JH121) to sections overnight at room temp at 1 in 500 (0.004mg/ml) using Dako diluent (S0809)
- Also apply negative control sera 1/250 (normal sera from same species as primary antibody) to control section and if possible use reagent control antibody that is well demonstrated in the tissue under investigation.
12. Wash sections well using PBS 3 x changes for 5 minutes each change
 13. Apply DAKO Envision + (Dako k4001) reagent to sections for 30 minutes
 14. Wash sections well using PBS 3 x changes for 5 minutes each change
 15. Make up DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer (wear gloves)
 16. Apply DAB to sections for 10 minutes
 17. Wash sections well using PBS for 5 minutes
 18. Rinse sections in running water
 19. Place sections in Mayers haematoxylin to elaborate nuclei, 30 seconds (or in Harris's for 2 minutes then differentiate with three dips in 1% acid alcohol).
 20. Rinse in running water
 21. Place in approx 400ml of water with 6-8 drops of ammonia for 30 seconds
 22. Rinse well in running water
 23. Dehydrate in 95% ethanol, then two changes of 100% ethanol (2 minutes each change)
 24. Clear in three changes of xylene (2 minutes each change) and mount sections in Depex (or similar)

TGF- β 1 ABCAM (ab27969) staining method:

1. Section paraffin blocks at 3 microns after cooling in -20-degree freezer or on ice blocks 5-10 minutes.

2. Pick up two sections separated by 40-50 microns (approximately 10 sections) on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides [in distilled water (milli Q water) bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade].
3. Dry overnight at 37 degrees in incubator.
4. Dewax sections in "xylene" 2 x 5 minutes each in fume hood
5. Hydrate to water using 100% ethanol followed by 95% then 70%, for 3 minutes each change
6. Rinse sections well in running tap water (2 minutes)
7. Place in 3% H₂O₂ in distilled water for 15 minutes.
8. Wash in running water for 2 minutes
9. Rinse in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) for 2 minutes
10. Block non specific protein using Dako serum free protein block (X0909) for 30 minutes
11. Apply ABCAM (cat no ab27969) (clone TB21) primary antibody to sections overnight at room temp at 1 in 16000 (0.0000625mg/ml) using Dako diluent (S0809)

Also apply negative control sera 1/1600 (normal sera from same species as primary antibody) to control section and if possible use reagent control antibody that is well demonstrated in the tissue under investigation.

12. Wash sections well using PBS 3 x changes for 5 minutes each change
13. Apply DAKO Envision + (Dako k4001) reagent to sections for 30 minutes
14. Wash sections well using PBS 3 x changes for 5 minutes each change
15. Make up DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer (wear gloves)
16. Apply DAB to sections for 10 minutes
17. Wash sections well using PBS for 5 minutes
18. Rinse sections in running water
19. Place sections in Mayers haematoxylin to elaborate nuclei, 30 seconds (or in Harris's 2 minutes then differentiate with three dips in 1% acid alcohol).

20. Rinse in running water
21. Place in approx 400ml of water with 6-8 drops of ammonia for 30 seconds
22. Rinse well in running water
23. Dehydrate in 95% ethanol, then two changes of 100% ethanol (2 minutes each change)
24. Clear in three changes of xylene (2 minutes each change) and mount sections in Depex (or similar)

Measurements and image analysis:

All quantitation of immunohistology end points was done by myself.

All slides containing tissue section samples of participants were coded by our laboratory manager and I was blinded to the diagnoses and time point during the whole process of measurements and analyses of results.

First, using x400 magnification of microscope (BH2, Olympus, Japan), as many pictures of non-overlying fields as possible were taken from the area of interest in tissue samples using a computerised camera (Spot Camera, Diagnostic instruments, inc., USA). For measurements related to the Rbm, the microscopic field was focused on the Rbm and for measurement of vessels in the LP, the microscopic field was focused on the LP. Then eight independent non-overlapping pictures were randomly selected for measurements. Those slides that did not have enough tissue for eight pictures were excluded from further analysis. Parts of the tissue that contained holes or folded tissue or were crushed were also excluded.

Tissue examination and measurements were performed using a computer assisted image analysis tool (Image-Pro version 5.1, Media Cybernetics, USA). This tool makes it possible to perform detailed examination of the pictures taken from tissue sections and to automatically measure of different structures. It is possible to measure automatically the length of linear or curvilinear objects and thickness and surface area of tissue compartments.

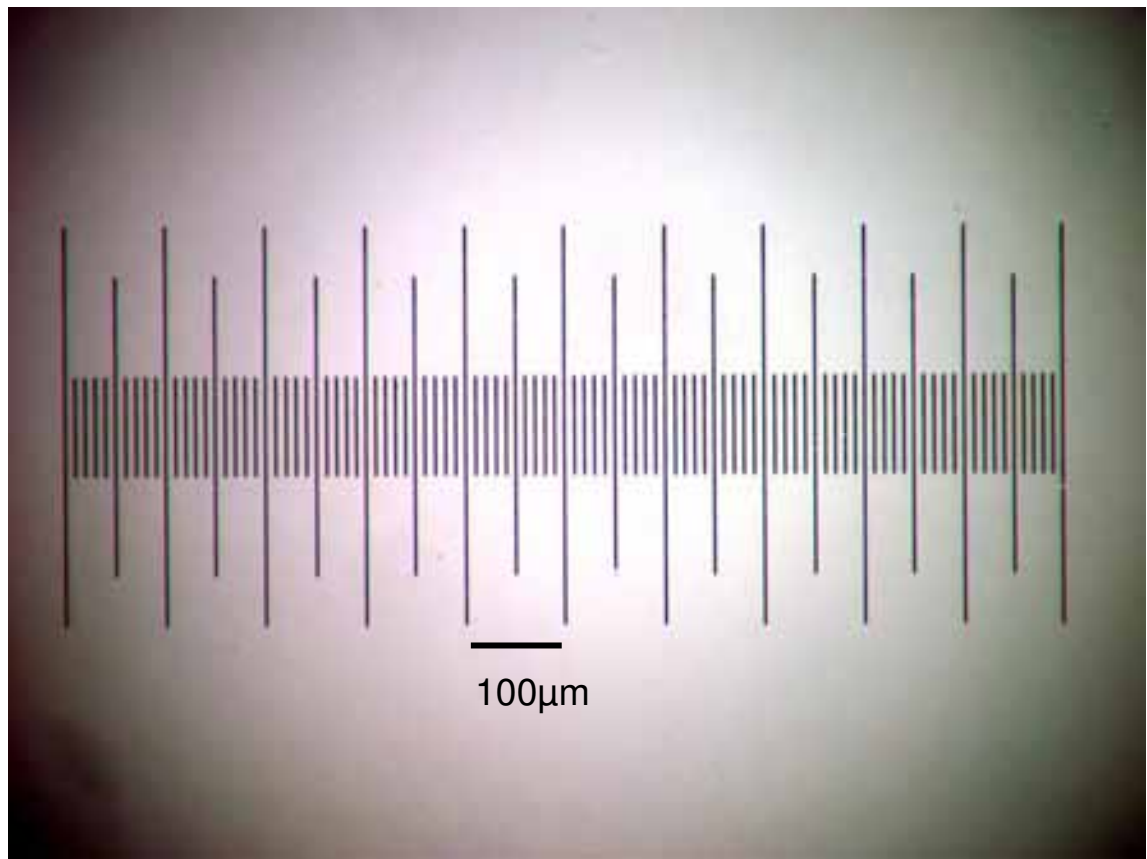


Figure 3-6. *Calibration ruler.* This picture is taken at x100 magnification. The whole length of the ruler is 1 mm (1000 μm). This ruler is used for calibration of measurements.

Using a calibration ruler, the image analysis tool was calibrated before use (Figure 3-6). Users can define the scale of measurements for the tool, e.g. mm or μm for length and mm^2 or μm^2 for surface area. Thickness of objects can be measured by delineating the borders around it.

It is also possible to measure tissue content of a specific molecule of interest by this tool using staining intensity. For example, we measured the intensity of the VEGF and also TGF- β immunostaining in different compartments of the tissues. After examining the tissue under the microscope, we define what colour we would like to be measured by the image analysis tool. Then the area of interest (e.g. the epithelium or the LP) is defined for the software (Figure 3-7). At this stage the tool is ready to measure the intensity of the immunostaining in the area of interest. The results were reported as the ratio of area stained compared to total area examined.

The Rbm stands beneath the epithelium and immediately below the true BM (Figure 3-8 and 3-9). The true BM contains Collagen IV and therefore stains with Collagen IV antibody. Although the Rbm does not contain Collagen IV it is clearly visible by Collagen IV antibody and hematoxylin counter-staining (Figure 3-10). It separates the epithelium from the LP. *Rbm thickness* was measured by delineating the outer and inner borders of the Rbm throughout the whole length of the microscopic fields. Then using the image analysis tool the average thickness of the Rbm was computed automatically (Figure 3-11), and the average of 8 fields was calculated for each subject.

To measure *Rbm splitting*, the length of the outer border of Rbm and the total length of Rbm splits were measured in each field (Figure 3-12). The whole visible length of the Rbm in the microscopic field was used for measurement. The total length of splits was summated and divided by the length of Rbm as denominator. Where the splits occurred in parallel layers in the same section of Rbm, all of them were included in the measurement (Figure 3-13). The results were reported as μm length of split per μm length of Rbm x 100.

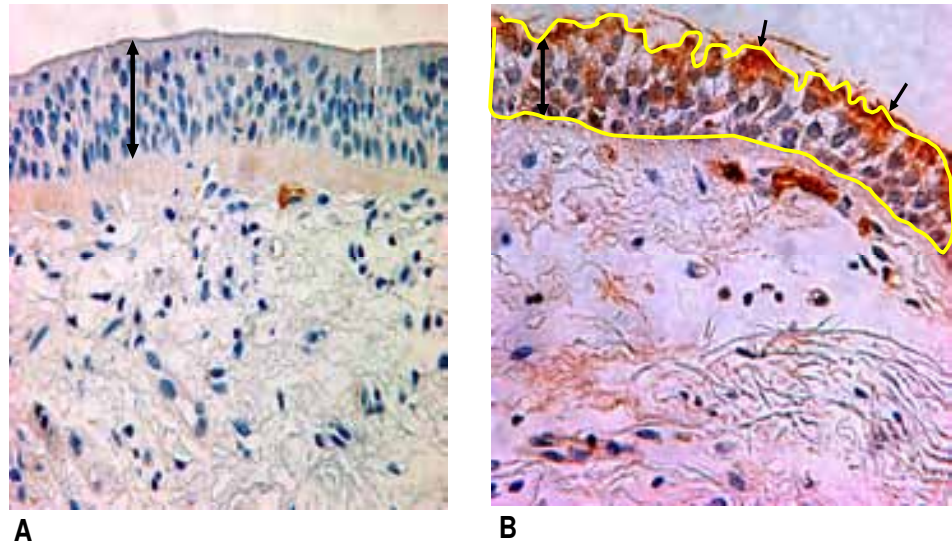


Figure 3-7. *Measuring VEGF staining.* The epithelium (two headed arrow) in **A** has almost no VEGF staining and in **B** is stained strongly for VEGF (arrows). Users define the colour to the image analysis tool and determine the area of interest (delineated here with yellow line). At this stage the tool is ready to automatically count and report the intensity of the stain as per unit surface area examined. VEGF staining, x400.

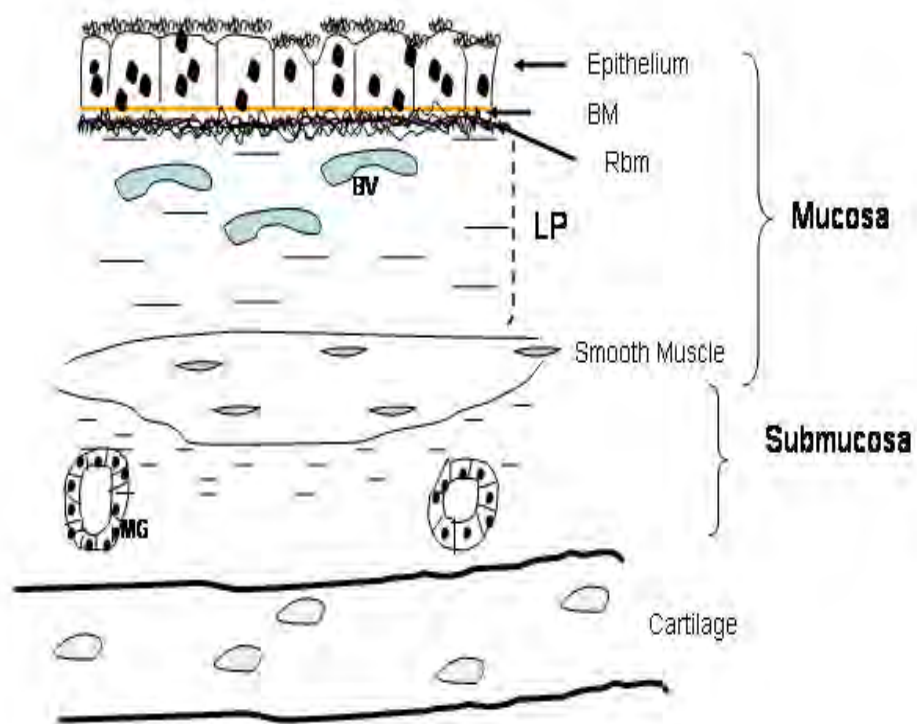


Figure 3-8. *Schematic presentation of bronchial wall structure.* Epithelium is the most superficial layer of the mucosa. The epithelium of airways are cylindrical, pseudostratified and ciliated. The cells seat on the BM. BM separates the epithelium from the LP. Immediately beneath the BM there is a layer of loose tissue called the Rbm, which is structurally quite different from BM. The LP consists of loose connective tissue and contains blood vessels (BV). Smooth muscle is the deepest part of the mucosa. Deep to the mucosa there is the submucosa with loose connective tissue and mucous glands (MG). Cartilage and adventitia are the most superficial components of the airway wall.

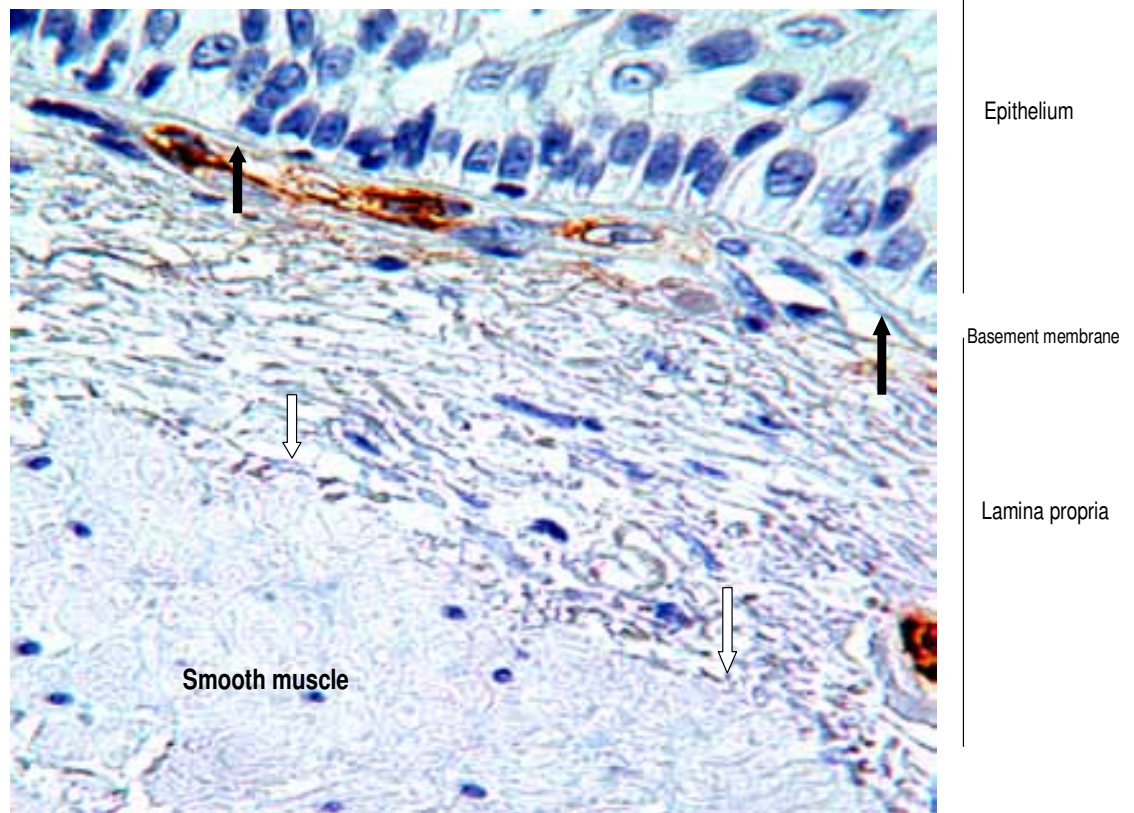


Figure 3-9. Different parts of the mucosa in a BB are shown here. The Rbm is marked by black arrows. Vessels are seen in the LP as tubular shadows. Factor VIII staining, x400.

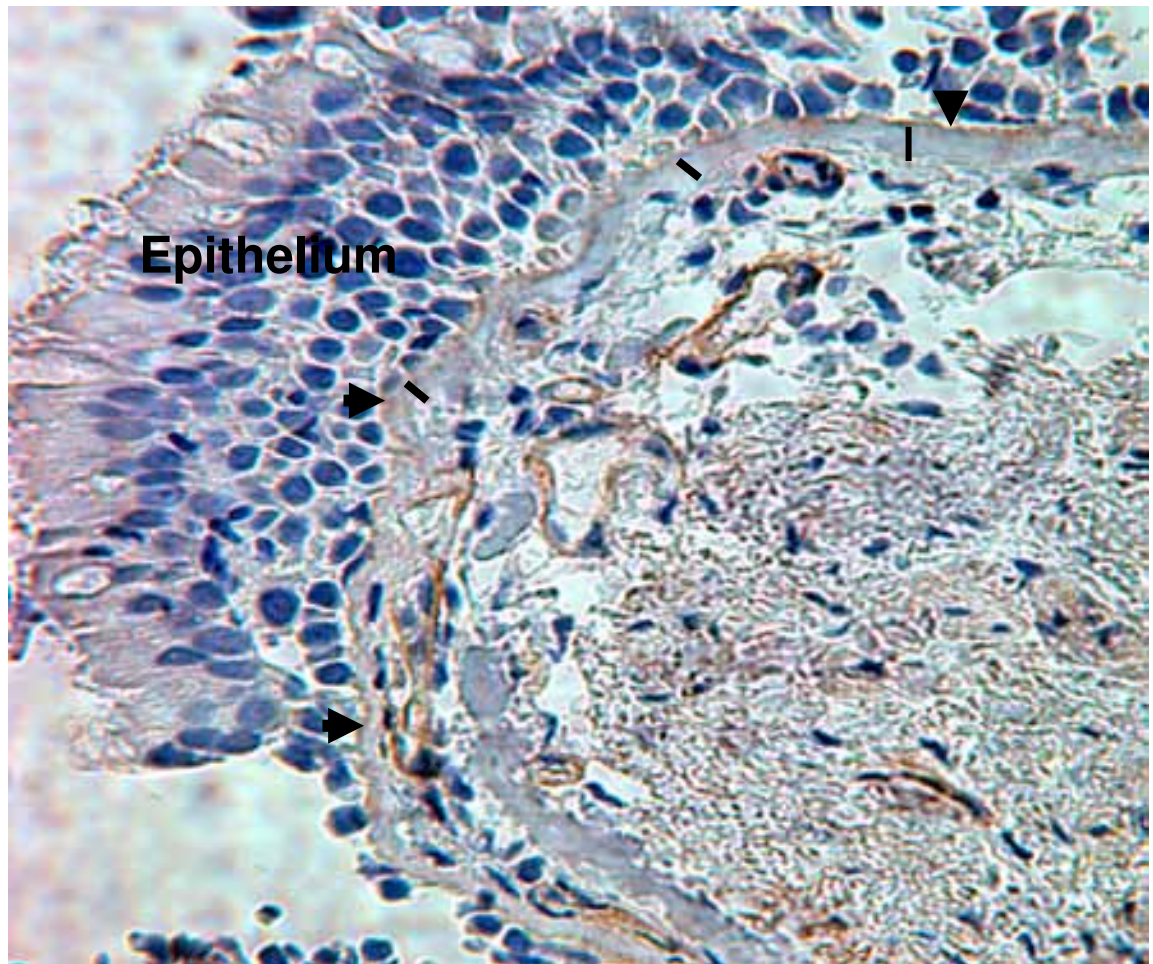


Figure 3-10. *Collagen IV staining of tissue.* The true BM is stained with collagen IV antibody (thick arrows). The Rbm is visible beneath the true BM and its thickness is marked with straight lines.

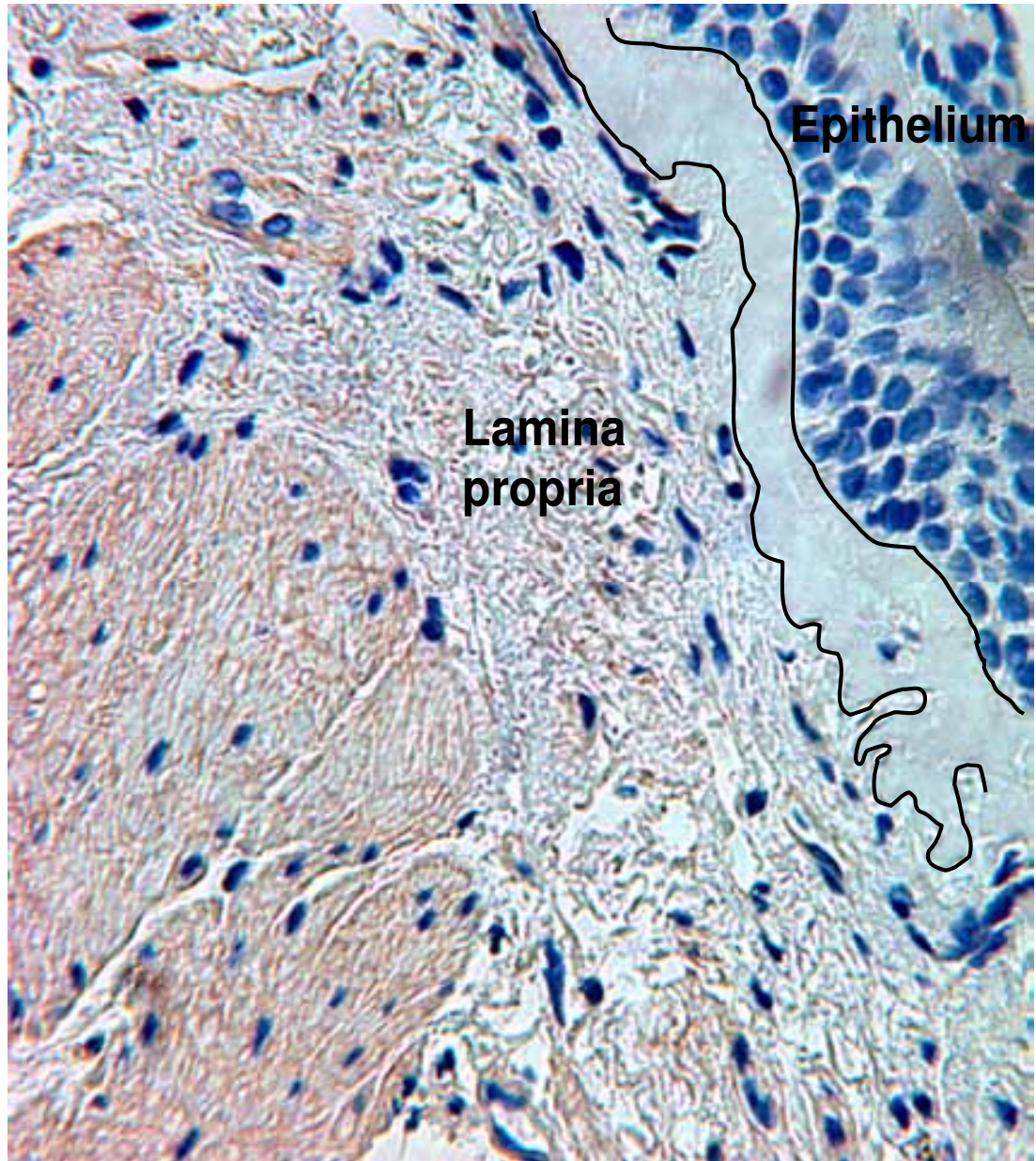
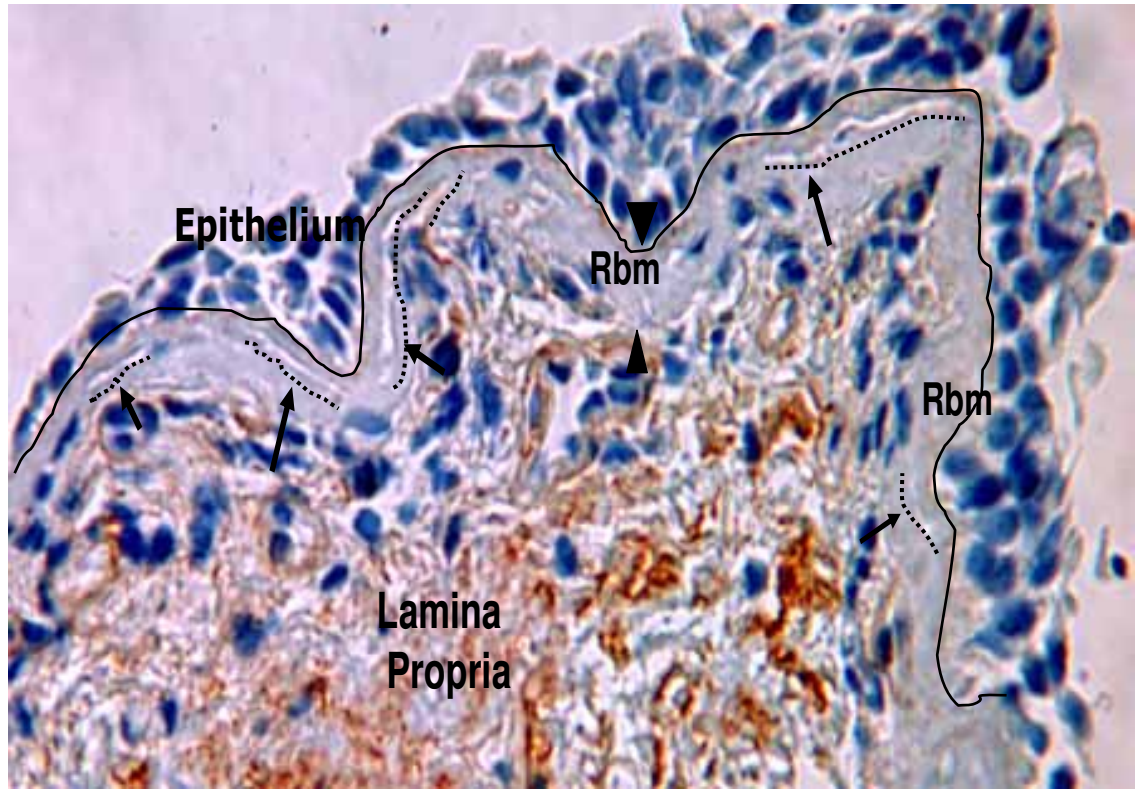


Figure 3-11. *Measuring Rbm thickness.* The Rbm is delineated bilaterally using image analysis tool. Then the average thickness is measured. Collagen IV staining, x400.



A

Figure 3-12. *Measurement of the length of Rbm splitting.* The outer border of the Rbm is marked with continuous line. The splits are marked by dotted lines. Collagen IV staining, x400

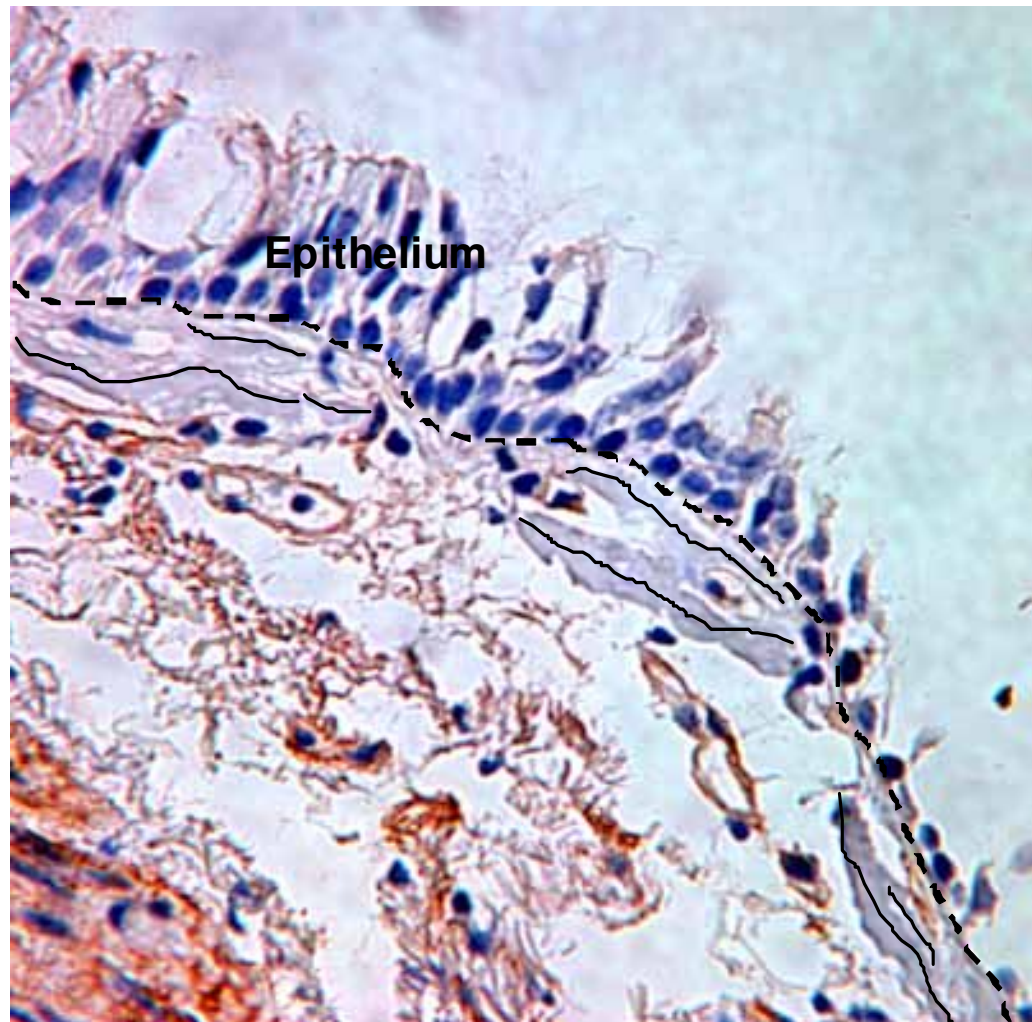


Figure 3-13. *Rbm splits in parallel layers.* The epithelium is attached to the true BM (dashed line). Splits are visible which lie in parallel (marked by continuous lines).

LP vessels were outlined by immunostaining (Collagen IV or Factor VIII). For our cross-sectional and longitudinal studies we used Collagen IV staining, as this had been previously used extensively by our group in studies of asthma and bronchiolitis obliterans syndrome (BOS) (B. N. Feltis et al., 2007; Bryce N. Feltis et al., 2006; Orsida et al., 1999; Orsida et al., 2001; L. Zheng et al., 1999).

Vessels that were embedded within the Rbm or had at least one border in contact with the Rbm were considered as Rbm vessels (Figure 3-14). All other vessels from the internal border of the Rbm up to the depth of 150 μm were considered as vessels in the LP (Figure 3-15). Using the image analysis tool, the area of the LP examined from the inner border of the Rbm up to 150 μm was measured. “Vascular area” was assessed as the area enclosed by the Collagen IV staining of the endothelial basement membrane (Figure 3-15). The total number and summated area of all vessels in the eight locations were measured using the image analysis tool. Measurements for Rbm and LP vessels were normalised by dividing by the length of Rbm and also the surface area of the LP examined, respectively. The number and area of vessels in the Rbm were reported as number per mm length of the Rbm and μm^2 per mm length of the Rbm; number and area of vessels in the LP were reported as number per mm^2 surface area of the LP and μm^2 per μm^2 surface area of the LP x 100.

To test my intra-observer repeatability, 12 slides were recoded by our laboratory manager (Mr. Steven Weston) and recounted by me. I was blinded to the diagnoses.

Statistical methods:

The data for coded and unblinded groups were analyzed using SPSS 16 for Windows. The first step of analysis was for the lab manager to uncode the data into groups, but to maintain my blindness to clinical groups until the analysis was completed.

The normality of the distribution of data variables was determined first.

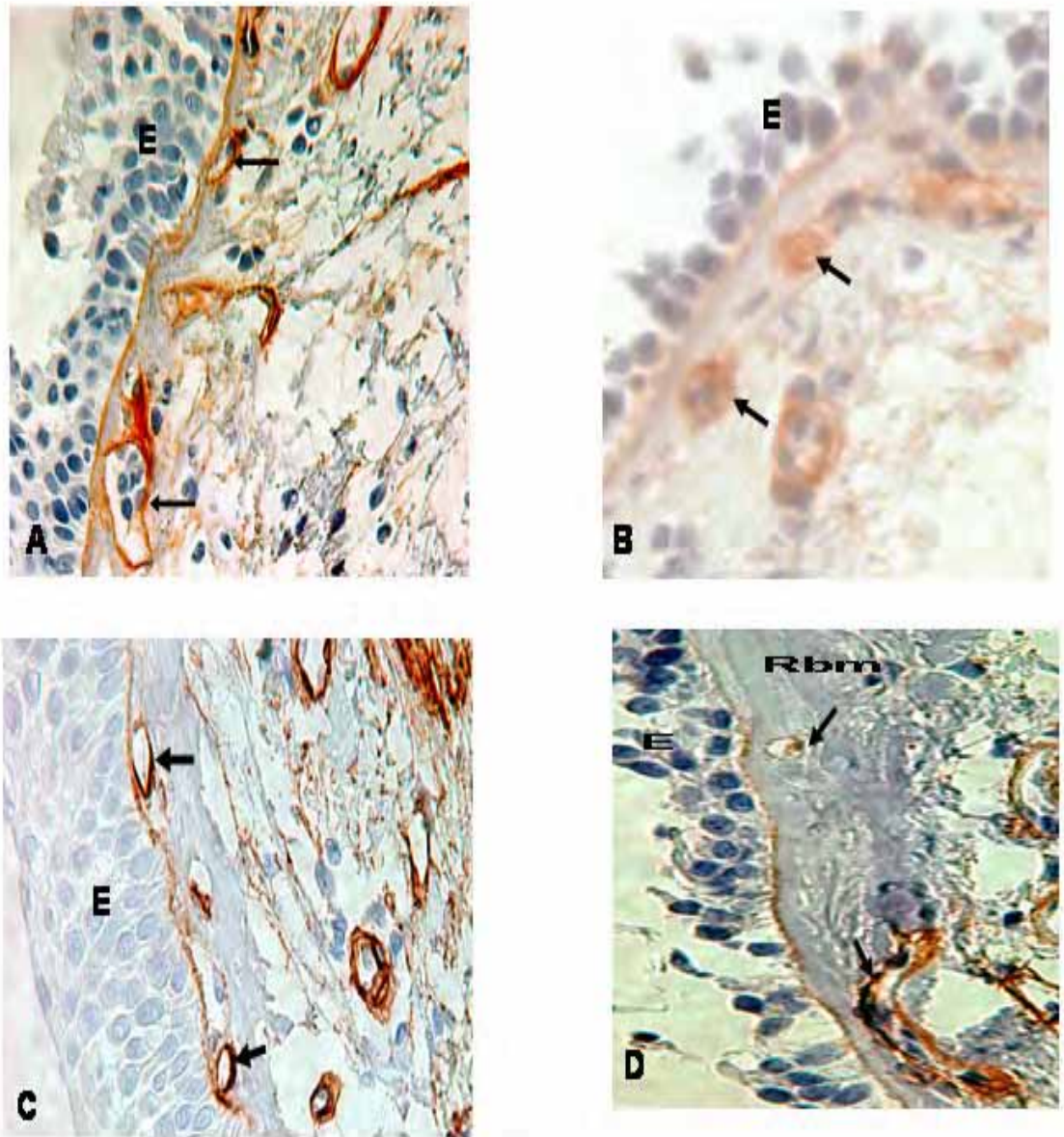


Figure 3-14. *Rbm vessels (arrows).* The epithelium (E) seats on the true BM. The Rbm is visible as a compact matrix beneath the BM. In A and B vessels can be seen in contact with the Rbm; they have at least one border in contact with the Rbm. In C vessels are embedded within the Rbm. In D there is a vessel within and a vessel in contact with the Rbm. Collagen IV staining, x400

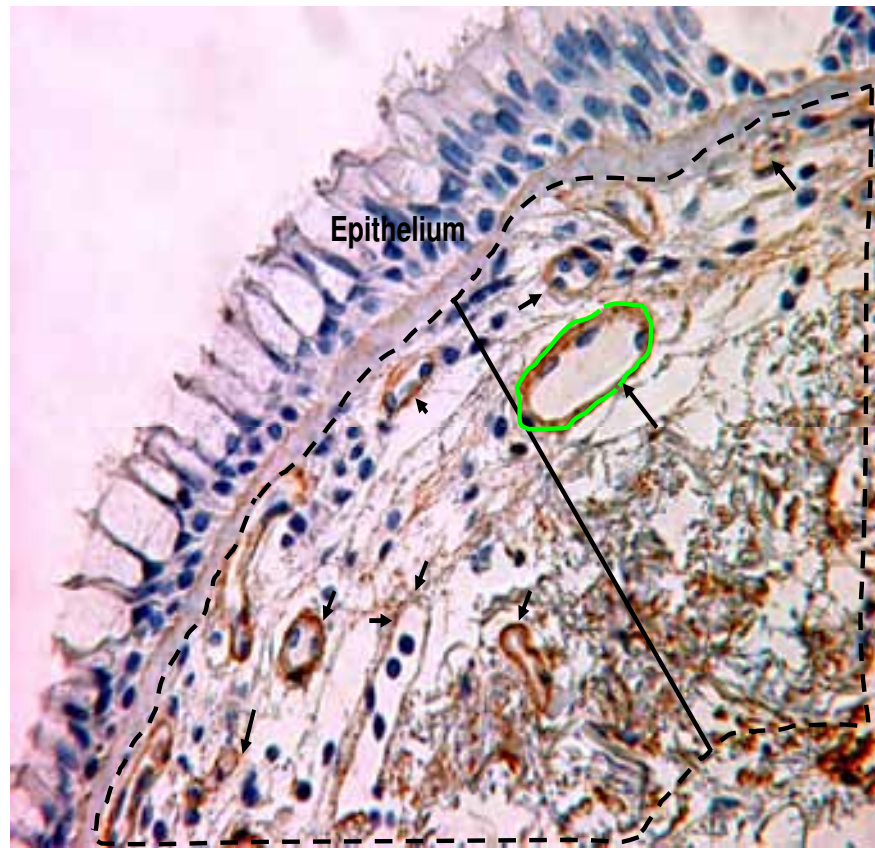


Figure 3-15. *Vessels in the LP.* The black straight line is drawn from the internal border of the Rbm to the depth of 150 μm of the LP. Vessels are delineated by collagen IV staining (arrows) and can easily be counted. Vascular area is measured as the area enclosed by collagen IV staining of the endothelial BM (the circumferential line around one vessel shows an example of vascular area measured). Dashed line surrounds the area of the LP that vessels were counted. Vessels in contact with the Rbm were counted as Rbm vessels and were not counted as LP vessels. Collagen IV staining, x400

To test the *difference between 2 groups*, Student's t-test was used for normally distributed variables. When *more than 2 groups* were compared, first ANOVA was used to test if there was any difference amongst the groups. If a significant difference was found, a t-test was used to compare the likely groups for difference as defined *a priori* in the study hypothesis. In a comparable way, Kruskal Wallis and Mann-Whitney U tests respectively were used to test these differences with *variables with non-normal distribution*.

For the longitudinal study, two *repeated measurements* to compare each outcome before and after treatment in the same group were compared with paired-samples Student's t-test for normally distributed variables and with Wilcoxon two-related-samples test for non-normally distributed variables. Both between groups and within group differences were tested. The differences between two groups were compared with independent t-test or Mann-Whitney test depending on the distribution of the variables. To compare the two groups for nominal variables, Chi-square test was used.

Pearson and Spearman correlation analyses were used respectively to find *correlations* between variables with normal and non-normal distributions.

Two-tailed p-values less than 0.05 were considered as *statistically significant*.

Group *results* throughout are reported as mean (\pm SD) or mean (with range) when the distribution of data was normal and as median (with range) when the data were non-normally distributed.

Power and sample size calculations:

Our prospective concepts of statistical power were based on previous experience of cellularity data in COPD and asthma, and of remodelling structural changes in asthma. These experiences uniformly suggested that we would obtain reasonably meaningful signals with numbers of 15-20 individuals. Retrospectively, we are now able to cross-check these assumptions with the data actually obtained.

Richmond et al have shown that power calculations suggest that 15 subjects are the optimal number for cellular quantitative studies on airway disease with little advantage for increasing beyond that number; as it avoids biological variability as confounding variable (Richmond, Booth, Ward, & Walters, 1996).

Our power calculations and sample size calculations with alpha error of 5% and beta error of 80% confirm their conclusions in the context of structural changes:

Cross-sectional study: For vessels in the LP, with our sample size (see results in Chapter Five, Cross-Sectional study), the study had greater than 90% power with alpha error = 5%. For Rbm thickness with alpha error = 5% and power = 80% a sample size just smaller than 15 was adequate. For Rbm splitting, with alpha error = 5% and power = 80% sample size smaller than 15 was adequate. For number of vessels in the Rbm with alpha error = 5% and beta error = 80% sample size of about 15 was adequate.

For the *longitudinal study*, the sample size calculations revealed number of subjects about 15 was adequate to find differences for Rbm splitting and Rbm vessels before and after treatment with alpha error 5% and beta error 80%. However, for vessels in the LP the study was under powered. With alpha error = 5% and beta error = 80% the sample size needed to find a significant difference was more than 50, though in fact there was no suggestion in the data that we had a Type-2 statistical error, i.e. it was extremely unlikely that any change occurred.

Repeatability and reproducibility:

Repeatability relates to intra-subject reliability. In this quality control method the same examiner evaluates the same slides twice but under blinded conditions to avoid bias.

Reproducibility relates to inter-observer agreement. Two examiners evaluate the same slides. Both examiners are blinded to the diagnoses and to the other's results (Warke et al., 2001).

We used the method for agreement published by Bland and Altman (J.M. Bland & Altman, 1986) to assess our repeatability and reproducibility. In the real world it is very unlikely for a researcher to produce the same results with repeated measurements. This method of evaluation of agreement indicates how clinically important and acceptable the differences between measurements may be (J. Martin Bland & Altman, 1996).

For repeatability, I counted 12 slides twice for all indices of interest, while they were coded and recoded by our laboratory manager (Mr. Steven Weston) and I was blinded to the diagnoses. For reproducibility, 24 slides were examined in different time periods by another independently-trained high-degree student and me. Our laboratory manager coded slides differently for each of the examiners and we were both blinded to the diagnoses and to each other's measurements.

The mean of the two measurements and the differences of the two measurements, and also the variance and the SD of differences were calculated. The standard deviation of repeated measurements can be used to estimate the size of errors. Coefficients of repeatability (CoR) were calculated by multiplying the SD by 1.96 (or making it simpler, by 2). (For normally distributed (Gaussian) differences, 95% of differences will lie between mean of differences ± 1.96 SD of differences. Measurement errors usually follow normal distribution; the measurements themselves do not have to follow this type of distribution). The CoR is the limit of differences that can occur in repeated measurements when there is no bias; 95% of differences are expected to be less than 2 standard deviations. In addition we would not expect the mean of differences being other than zero (in reality they were close to zero). The 95% limits of agreement (LoA) were calculated as [mean of differences $\pm 2 \times$ SD of differences]; which would be almost equal to CoR when the mean of differences is close to zero (J.M. Bland & Altman, 1986; J. Martin Bland & Altman, 1996; Richmond et al., 1996; Warke et al., 2001).

A scatter plot of differences of measurements (Y axis) against means of measurements (X axis) was drawn using a Microsoft Excel spreadsheet containing the data. For

Table 3-1. Intra-observer agreement*

	Mean (range) 1 st reading	Mean (range) 2 nd reading	Mean of differences	CoR	95% LoA	CoR/mean x 100
Rbm splitting $\mu\text{m}/\mu\text{m}$ Rbm x 100	6.4 (0.0-24.5)	9.8 (0-28.8)	-3.3	9	-12.3 to 5.7	100%
Number of Rbm vessels/mm Rbm	7.3 (0.1-15.4)	8.1 (0.7-20.5)	-0.8	6.4	-7.2 to 5.6	83%
Area of Rbm vessels $\mu\text{m}^2/\text{mm}$ Rbm	670 (120- 1450)	780 (120- 2100)	-110	702	-810 to 590	97%
Number of LP vessels/mm ² LP	373 (148-544)	440 (140-624)	-31	248	-279 to 217	63%
Area of LP vessels $\mu\text{m}^2/\mu\text{m}^2$ x 100	5.3 (0.0-10.3)	6.3 (1.7-10.9)	-1	3.6	-2.7 to 4.5	62%

* CoR coefficient of repeatability, LoA limits of agreement

Table 3-2. Inter-observer agreement*

	Mean (range) 1 st observer	Mean (range) 2 nd observer	Mean of differences	CoR	95% LoA	CoR/mean x 100
Area of LP vessels $\mu\text{m}^2/\mu\text{m}^2$ x 100	4.1 (1.8-7.4)	3.7 (2.0-7.0)	0.4	4.2	-3.8 to 4.6	100%

* CoR coefficient of repeatability, LoA limits of agreement

reasonably repeatable measurements most of these dots should be located within \pm of the coefficient of repeatability (J.M. Bland & Altman, 1986).

Results: Although this is not a results chapter, I have decided to consolidate all the relevant material regarding agreement together. Table 3-1 summarises the results of repeatability tests on the cross-sectional study, and Table 3-2 presents the result of reproducibility test.

Figures 3-16 to 3-18 shows the scatter plots of means of measurements against differences of measurements for respectively: Rbm splitting, Rbm vessels and LP vessels. Figure 3-19 shows the same plot for our inter-observer reproducibility test. These figures show that most differences between first reading and second reading lie within \pm CoR.

We also tested *reliability* by intra-class correlation coefficients (ICC 3, 1), which varied from 0.83 to 0.97 and therefore are in the accepted range in the literature (Bacci et al., 2002).

Discussion: As expected, intra- and inter-researcher agreements were not perfect as the mean of differences between two readings were not zero. But the mean differences for most measurements were small (Tables 3-1 and 3-2); and almost all differences were within the 95% LoA (Figures 3-16 to 3-19). Overall, expectations for good agreement are where 95% of differences lie between 95% LoA.

To an extent, interpretation of the plots is subjective; there is no absolute number to define the presence or absence of good agreement (J.M. Bland & Altman, 1986). For some measurements, such as measuring blood pressure or core body temperature, it is possible to determine whether differences within the LoA are clinically or biologically significant or not. But for our measurements, it is hard to define clinically or biologically important limits. However, CoR for our intra- and inter-observer agreements was between 62-100% which is better than, or at least equal to, previous analyses of cellular data on airway biopsy material (Richmond et al., 1996). This indicates what degree of

change one can pick up over time or with an intervention and our data indicate that we can pick up changes that are biologically relevant i.e. less than halving to doubling of end points of interest. Thus, we conclude that our measurements had acceptable inter- and intra-observer agreements.

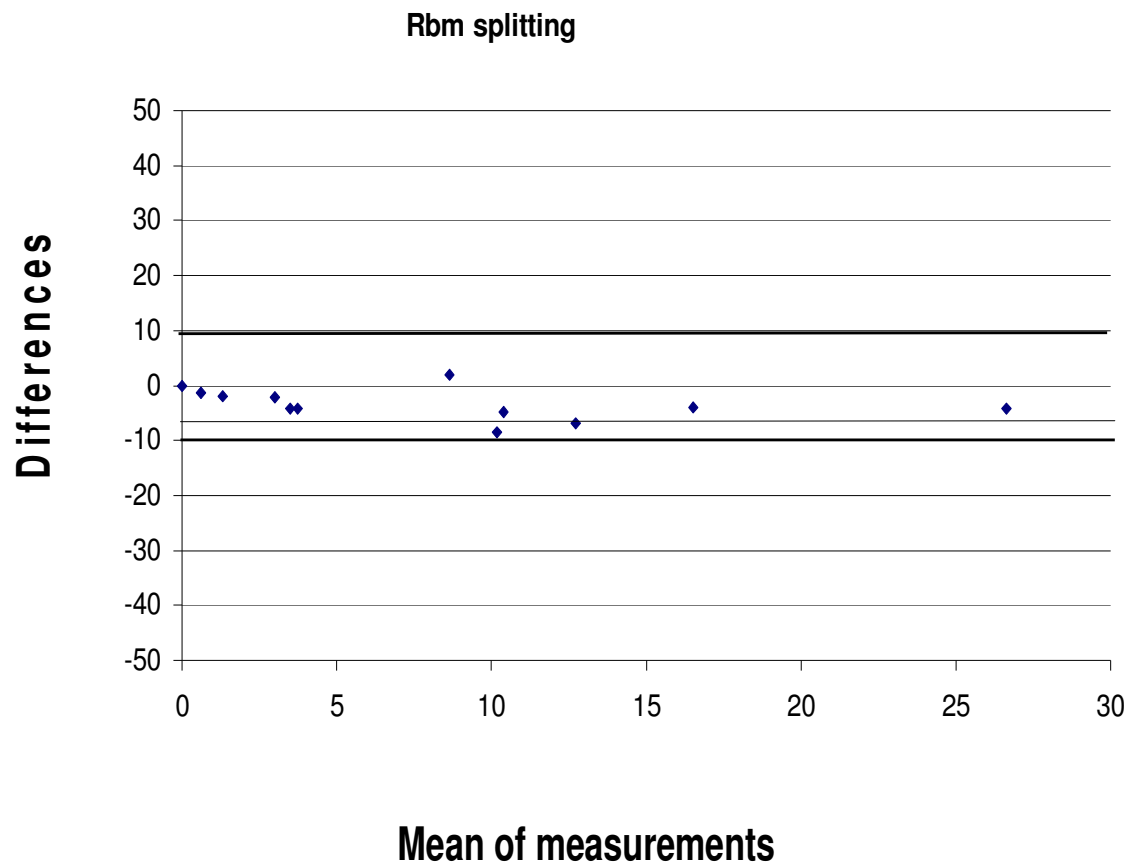


Figure 3-16. *Repeatability test for Rbm splitting.*

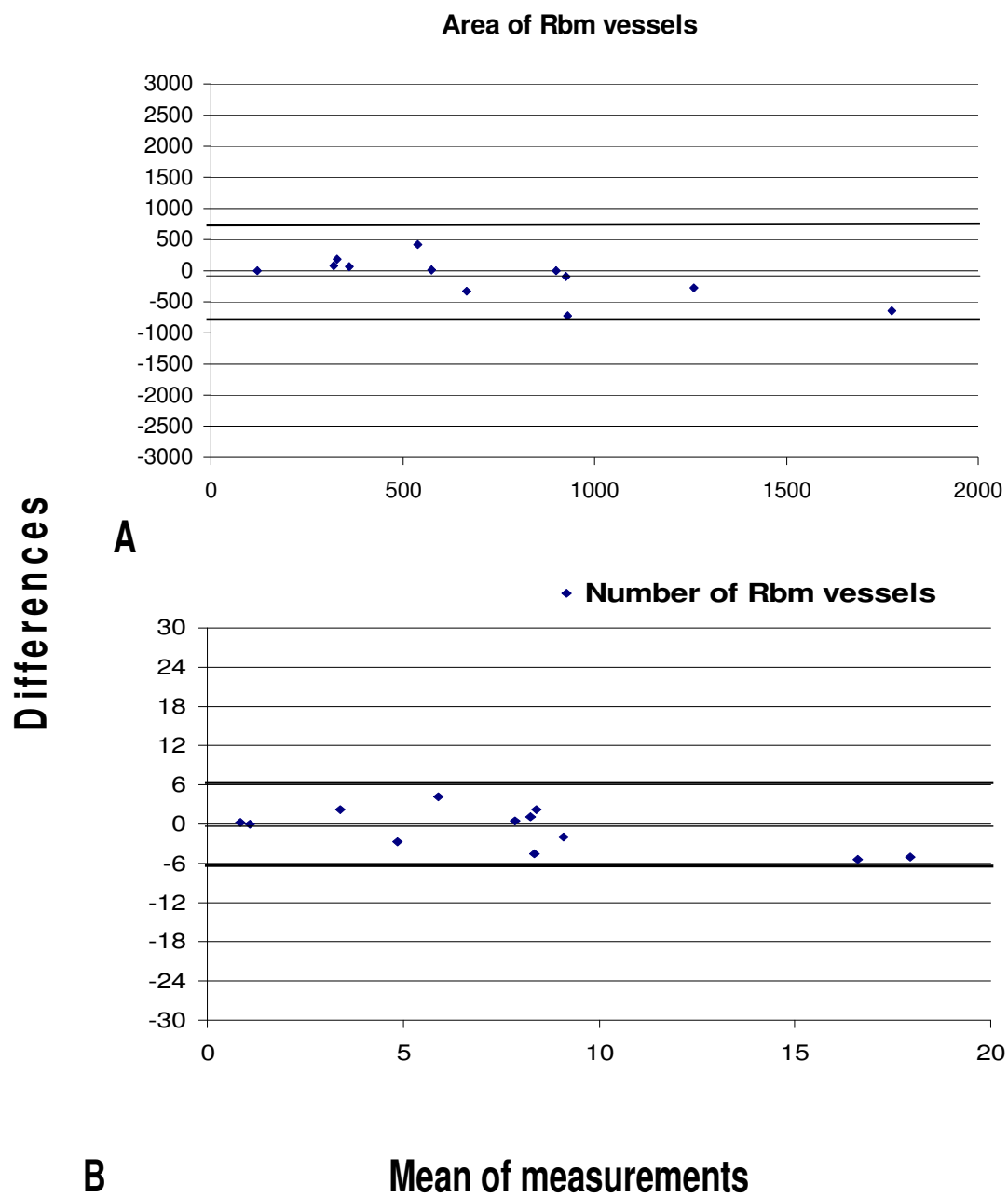


Figure 3-17. *Repeatability tests for A. area of Rbm vessels and B. number of Rbm vessels*

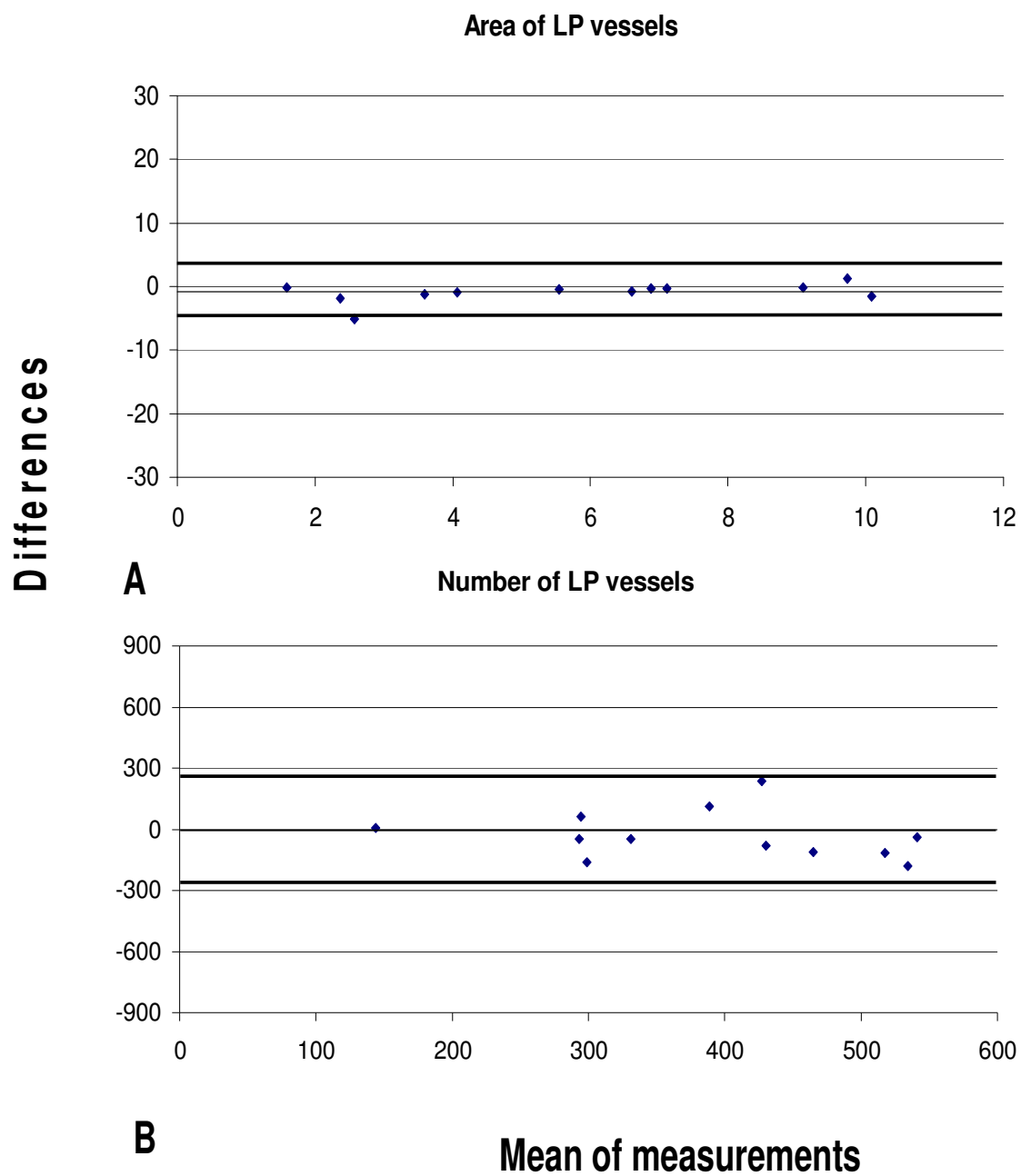


Figure 3-18. *Repeatability tests for A. area of LP vessels and B. number of LP vessels*

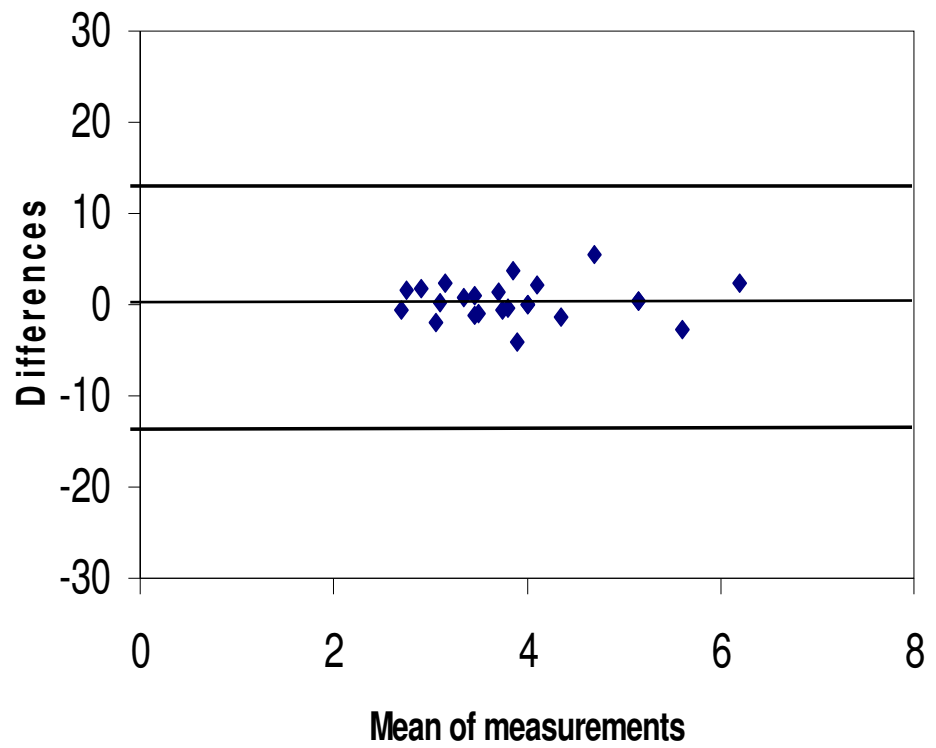


Figure 3-19. *Reproducibility test for area of LP vessels.*

Chapter Four

Material and Methods, Part Two: Histochemical Staining of Vessels; Collagen IV versus Factor VIII in COPD versus Normal Airways

Introduction:

Staining for blood vessels in the airway wall was such an important part of my thesis that I decided to specifically study and compare quantitatively my 'normative' stain (anti-Collagen IV) with the most frequently used alternative (anti-Factor VIII, an endothelial cell marker). The hypothesis was that anti-Collagen IV would stain more mature vessels than anti-Factor VIII, and that there would therefore be differences in vessel profiles when both were used.

Background:

Angiogenesis is under vigorous study in many diseases including inflammatory diseases and malignancies. Chronic inflammatory diseases of the airways such as asthma and COPD are no exceptions. During the last two decades many reports about vascularity of the mucosa in asthma have been published (E. H. Walters, Soltani, Reid, & Ward, 2008). There are only a few reports on vascularity in COPD.

Investigations have shown that hematoxylin and eosin staining alone is not efficient in detecting vessels in tissue and that there is a need to add specific immunostaining to tissue to see vessels (Barsky, Baker, Siegal, Togo, & Liotta, 1983). Besides, some structures such as lymphatic vessels are indistinguishable from blood vessels in tissue sections without using specific markers.

Blood vessel walls consist of three layers: intima, media, and adventitia. Intima contains a layer of squamous epithelial cells (endothelial cells) that rest on the BM which they secrete. Deeper to the BM there is the subendothelial connective tissue layer. The media contains smooth muscle cells. The adventitia, which is the outermost layer of the vessel wall, consists of collagenous tissue. Capillary walls are thin and contain endothelium, endothelial BM and a surrounding loose network of collagen and reticular fibers. Endothelial cells store Factor VIII in Weibel-Palade bodies that are membrane bound organelles (Mitchell & Schoen, 2010; Ovale & Nahirney, 2008a).

Lymphatic capillaries are abundant in connective tissue in the mucosa of the respiratory system. They are hard to differentiate from blood vessels by light microscopy. Their wall consists of just a thin layer of endothelial cells, the BM is incomplete or absent, providing more permeability than in blood vessels (Karkkainen, Makinen, & Alitalo, 2002; Mitchell & Schoen, 2010; Ovale & Nahirney, 2008b; Rutkowski, Boardman, & Swartz, 2006; Sacchi, Weber, Agliano, Cavina, & Comparini, 1999). Lymphatic endothelial cells are CD34 negative and express Factor VIII heterogeneously. Lymphatic markers such as transmembrane mucoprotein podoplanin, lymphatic vessel endothelial hyaluronan receptor-1 (Lyve-1) and VEGFR-3 can be used to differentiate lymphatic endothelial cells (Emirena et al., 2006; Fina et al., 1990; Karkkainen et al., 2002). VEGFR-3 is predominantly expressed in the lymphatic vessels and podoplanin, a podocyte cell surface mucoprotein, is expressed selectively by lymphatic endothelial cells (Karkkainen et al., 2002).

A small number of histochemical vessel markers are commonly used for blood vessel detection. An optimal marker should be specific for vascular endothelial cells,

independent of pathological changes in tissue (e.g. inflammation, malignancy, hypoxia, ischemia, shearing stress), resistant to usual methods of tissue fixation and processing, open to detection of different sizes of vessels (i.e. large and small) and be able to detect different types of vessel endothelial cells, i.e. capillary, vein, arteriole and arteries. It has been shown that under pathological conditions, endothelial cells modify their antigen presentation (Pusztaszeri, Seelentag, & Bosman, 2006), and most available histochemical markers do not fully meet these characteristics, but the pros and cons of different immunohistologic antibody systems has not been worked out in any detail.

The most commonly used tissue vessel markers in studies of the respiratory system have been antibodies against Collagen IV (Figure 4-1) and Factor VIII (Von Willebrand factor) (Figure 4-2), CD31 (EN-4) (platelet/endothelial cell adhesion molecule, PECAM) and CD34 (a cell surface glycoprotein that possibly acts as an adhesion factor on hematopoietic progenitor cells and vascular cells). Glycol methacrylate (GMA) processing and paraffin-embedding are superior to other methods for investigation of vessels in tissue samples (Vrugt et al., 2000).

Factor VIII antigen is produced by endothelial cells and is physiologically involved in platelet aggregation and adhesion (Martin et al., 1997; Sehested & Hou-Jensen, 1981). A number of studies have reported Factor VIII antibody to be a reliable marker for vessel detection (Harach, Jasani, & Williams, 1983). However, it has also been reported that Factor VIII antibody stains megakaryocytes, mesenchymal tissue and immune cells in addition to endothelial cells (Vrugt et al., 2000) (Parums et al., 1990). Further, Factor VIII is not present on all endothelial cells but is also present on lymphatic endothelium (Horak et al., 1992; Parums et al., 1990). The capability of Factor VIII antibody to detect blood vessels has also been shown to be related to the size of vessels. One study on primary breast cancer found that Factor VIII antibody lost its sensitivity to mark vessels as they increase in size (Bettelheim, Mitchell, & Gusterson, 1984). In contrast, another study found that Factor VIII antibody could not stain capillaries but stained large vessels (Pusztaszeri et al., 2006).

Our group has had substantial experience in using Collagen IV antibody as a blood vessel marker in BB and has successfully published the results (B. N. Feltis et al., 2007; Bryce N. Feltis et al., 2006; Orsida et al., 1999; Orsida et al., 2001). With that experience in mind, I decided to mainly use Collagen IV antibody to investigate vascular remodelling in the current studies reported in this thesis.

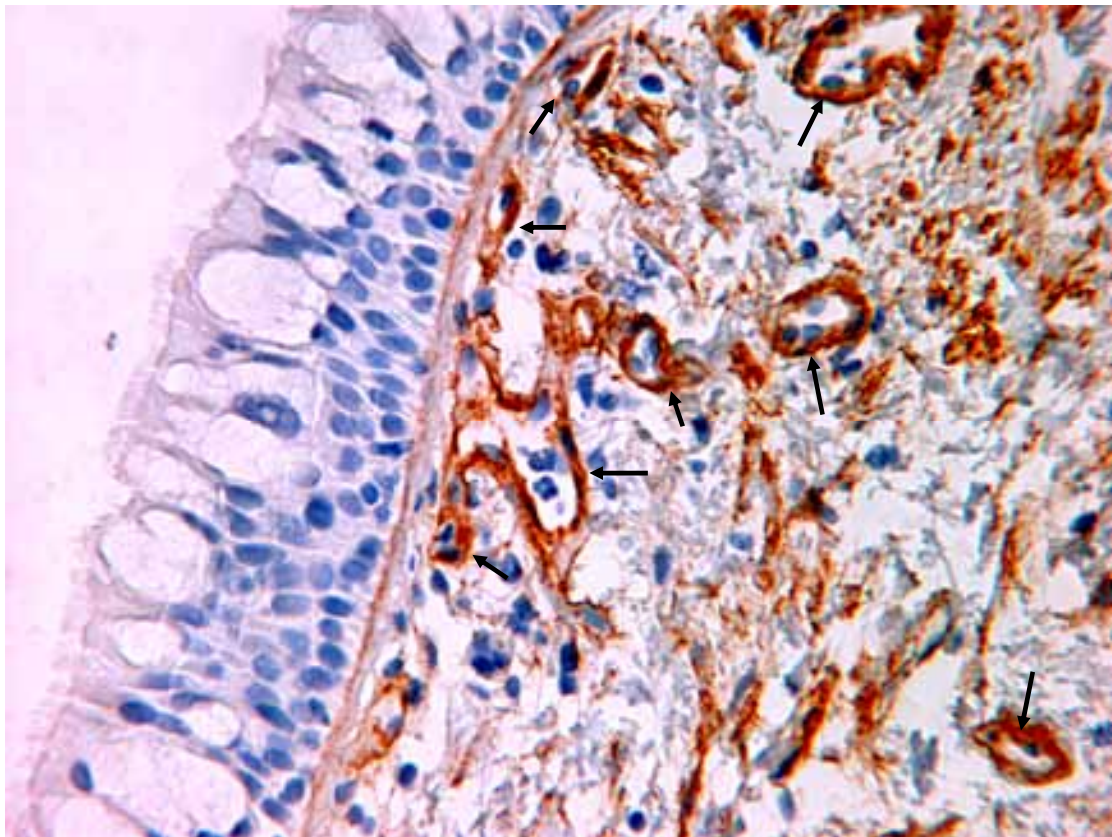


Figure 4-1. *Vessels in the LP are marked by collagen IV antibody (arrows). x400*

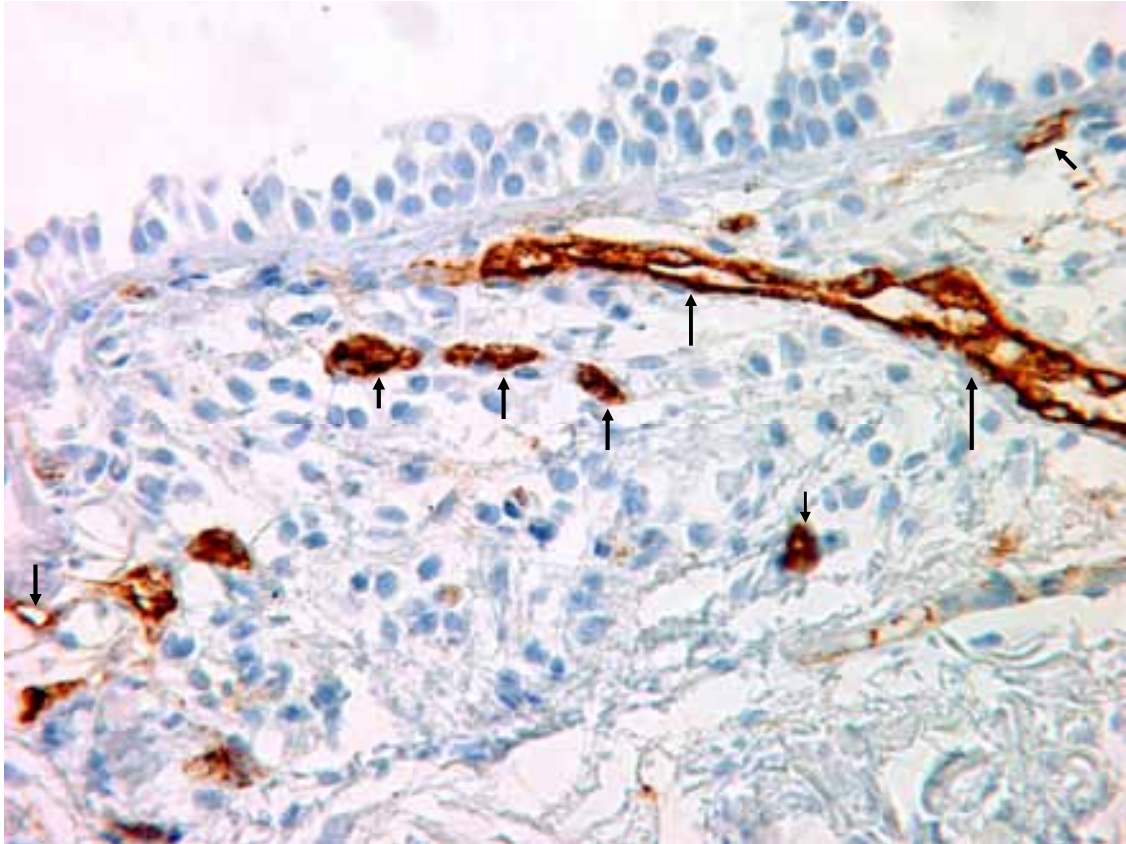


Figure 4-2. *Vessels in the LP stained by factor VIII antibody (arrows). x400*

Collagen IV antibody delineates endothelial basement membrane (Barsky et al., 1983; Vrugt et al., 2000). Barsky et al. reported that Collagen IV antibody is a sensitive marker of capillary endothelial cells and that it does not stain lymphatic capillaries. They supported this by using dye to detect lymphatics, which were completely similar morphologically to blood vessels under microscopic examination (Barsky et al., 1983). However, another group, in a study on breast cancer, found that Collagen IV antibody delineated the basement membrane of both blood and lymphatic vessels whereas Factor VIII antibody did differentiate between small vessels and lymphatics (Bettelheim et al., 1984).

CD31 and CD34 antibodies detect both immune cells and endothelial cells (Vrugt et al., 2000). CD31 is a member of the immunoglobulin superfamily and a cell surface glycoprotein. It is also present on inflammatory cells (Pusztaszeri et al., 2006) and is heterogeneously expressed in the lymphatic endothelium (Karkkainen et al., 2002). CD34 antibody detects a cell surface antigen on hematopoietic progenitors, and also binds to capillary endothelial cells in normal and tumour tissues. CD34 protein is probably involved in endothelial cell migration. Anti CD34 antibody can also stain some mesenchymal tissue components and neoplastic cells. Endothelial cells of veins and arteries of larger size were CD34 negative in a report by Fina et al. (Fina et al., 1990). Even so, some investigators have preferred using CD34 antibody for their studies as they found it to be more reliable than other markers, at least in neoplastic diseases (Edwards et al., 2001; Hansen et al., 2000).

Martin et al. found anti-CD34 antibodies to be the most sensitive markers for vessels (Martin et al., 1997), but they reported that the CD31 antibody was unreliable because at stronger concentrations it stained inflammatory cells and endothelial cell detection was thereby obscured.

Horak et al. reported that anti-CD31 antibody was the most sensitive vessel stain marker in their study on primary breast cancer (Horak et al., 1992).

Antibodies to Collagen IV and Factor VIII stain different structures in vessels and the literature indicates that markers for immunostaining of vessels do not uniformly detect vessels of varying sizes and can vary between vessels in different pathologies. I therefore decided to compare the efficacy of Collagen IV and Factor VIII antibodies as markers for blood vessels in BB from COPD and normal subjects, and investigate what the differences are in the vessel profiles that they stain.

Material and methods:

BB taken from segmental airways from 7 healthy nonsmokers (H-N) and 28 COPD subjects (15 current smokers and 13 ex-smokers) were cut at 3 microns and stained with Factor VIII monoclonal antibody (Dako M0616, Dako Cytomation, Denmark, dilution: 1 in 150) and Collagen IV monoclonal antibody (Dako M0785, Dako Cytomation, Denmark, dilution: 1 in 150). Details of subject recruitment, tissue processing, histochemical staining and measurements can be found in Chapter Three, Material and Methods.

Measurements: The measurements were done by me after slides were coded by our laboratory manager, Mr. Steven Weston, and I was blinded to the diagnoses. Vessels down to a depth of 150 μm of the LP from the internal (antiluminal) border of the Rbm were included in the measurements. Number and area of vessels were measured with the help of the computer-assisted image analysis system described in Chapter Three. These data were normalised by dividing by the surface area of the LP examined. Mean vascular size (MVS) of vessels was measured as total area/number of vessels. Mucous glands and muscle were excluded from measurements in the LP.

Results:

Thirty five subjects participated in the study. Table 4-1 summarises the demographics. Of the 28 COPD subjects, 15 were current smokers and 13 were ex-smokers.

The data from the two methods of vessel staining were first tested for agreement using Spearman's correlation (Figures 4-3 to 4-5). The correlation coefficients for number and

Table 4-1. Demographics

Groups (number)	H-N (7)	COPD (28)
Age*†	59 (32-68)	61 (46-78)
Gender female /male‡	2/5	9/19 (32%)
Pack-years of smoking*	0	47 (18-148)

*Median (range)

†P = 0.19 NS (independent samples t-test)

‡P = 0.06 NS (Chi-square test)

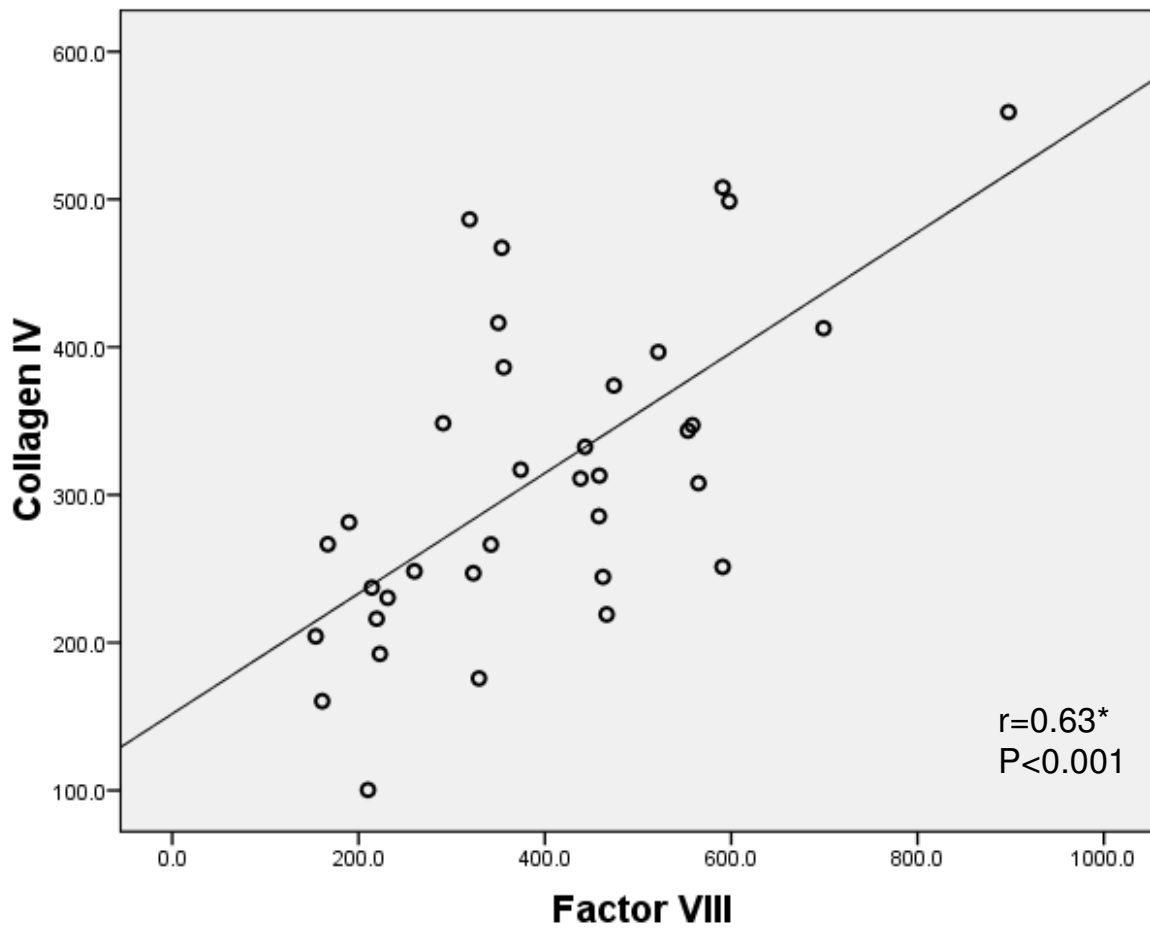


Figure 4-3. *Number of vessels/mm² of LP examined by the two methods. *Spearman's correlation coefficient.*

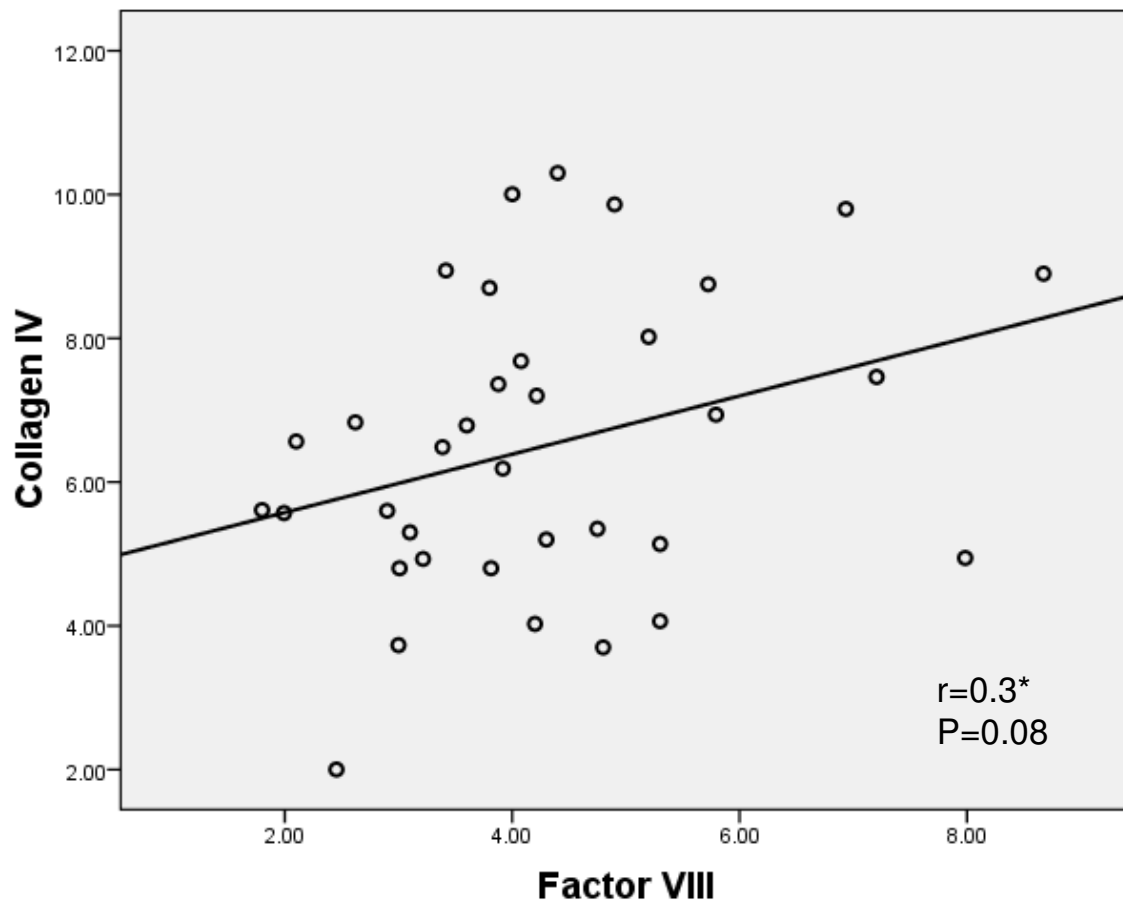


Figure 4-4. Area of vessels $\mu\text{m}^2/\mu\text{m}^2$ of the LP examined by the two methods.

*Spearman's correlation coefficient.

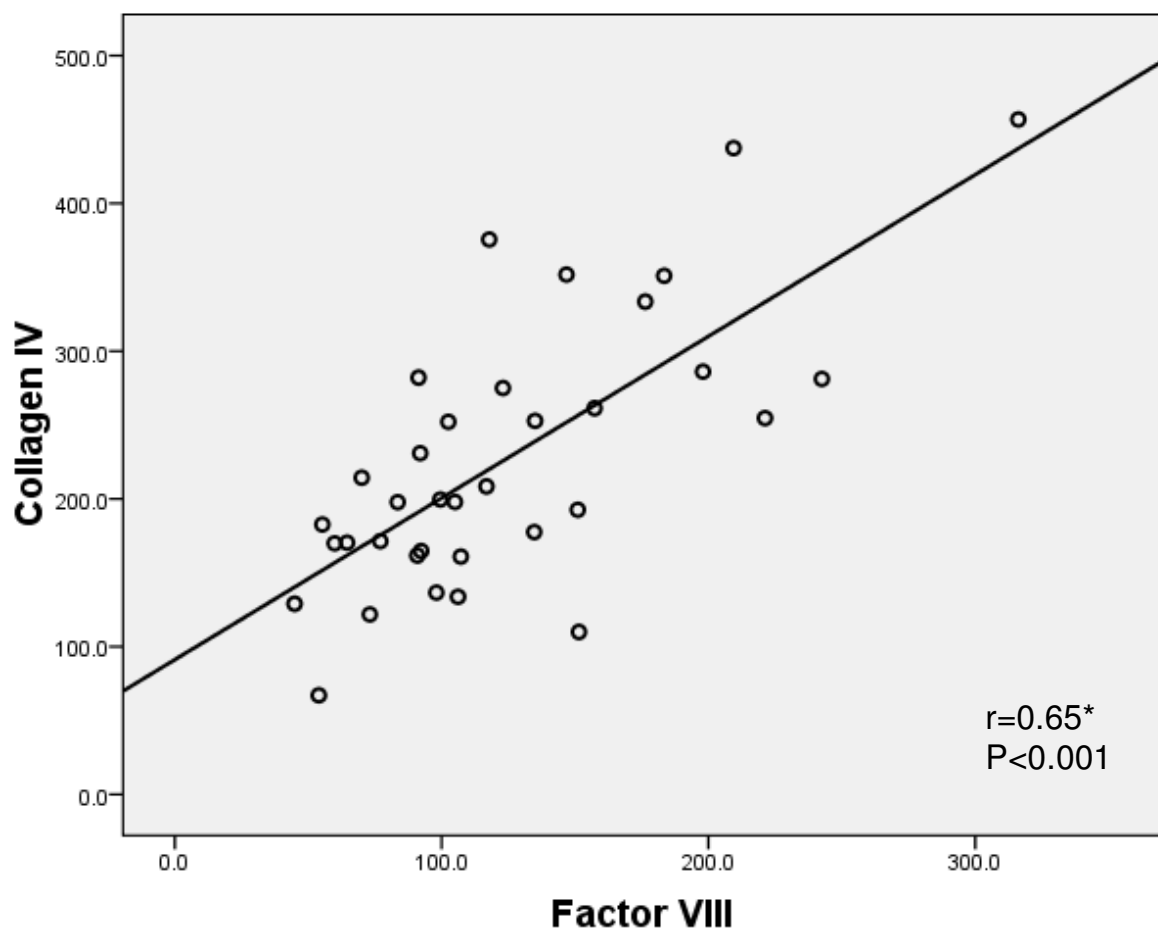


Figure 4-5. *MVS examined by the two methods.* *Spearman's correlation coefficient.

area of vessels and MVS were 0.63 ($p < 0.001$), 0.3 ($p = 0.08$) and 0.65 ($p < 0.001$) respectively.

Then, the *agreement* between the two antibodies was examined using the method reported by Bland and Altman (J.M. Bland & Altman, 1986) (see Chapter Three for details). Very briefly, by this method the means of the two measurements were plotted against the differences of the two measurements. Coefficient of repeatability (CoR calculated as SD of differences $\times 2$) and 95% limits of agreement (LoA = mean of differences ± 2 SD of differences) were calculated for every measurement (Figures 4-6 to 4-8 and Table 4-2). CoR/mean $\times 100$ for number and area of vessels and MVS were 88%, 115% and 139% respectively.

Area of vessels (Tables 4-3 to 4-5 and Figures 4-9 to 4-11) with Factor VIII staining was significantly less than with Collagen IV staining in both COPD and H-N groups together [median (range) 4.0 (1.8-8.7) vs. 6.5 (2.0-10.3), $p < 0.001$], COPD group [median (range) 4.1 (1.8-8.0) vs. 6.0 (2.0-10.3), $p < 0.001$] and H-N group [median (range) 3.9 (2.6-8.7) vs. 6.8 (3.7-8.9), $p < 0.05$].

MVS (Tables 4-3 to 4-5 and Figures 4-9 to 4-11) was significantly less with Factor VIII staining than with Collagen IV staining for both COPD and H-N groups together [median (range) 106 (45-316) vs. 200 (67-457), $p < 0.001$], COPD group [median (range) 112 (60-316) vs. 204 (110-457), $p < 0.001$] and H-N group [median (range) 70 (45-198) vs. 183 (67-286), $p < 0.05$].

When COPD subjects were compared to H-N controls, COPD had significantly fewer vessels in the LP with Collagen IV antibody staining [median (range) 266 (100-508) vs. 348 (311-559), $p < 0.05$] and also with Factor VIII antibody staining [median (range) 346 (154-598) vs. 474 (291-898), Student's t-test for log transformed data $p < 0.03$] (Tables 4-6 and 4-7); this significant difference was present between S-COPD and H-N [median (range) 329 (154-591) for S-COPD vs. 474 (291-898) for H-N, Student's t-test for log

Table 4-2. Agreement between two methods of vessel staining*

	Mean (range) Collagen IV	Mean (range) Factor VIII	Mean of difference s	CoR	95% LoA	CoR/ mean x 100
Number of vessels/ mm ² LP	313 (100-559)	395 (154-898)	83	310	-393 to 227	88%
Area of vessels μm ² /μm ² of LP x 100	6.5 (2.0-10.3)	4.3 (1.8-8.7)	2.2	6.2	-4 to 8.4	115%
Mean vascular size μm ² / number	227(67- 457)	124 (45-316)	103	245	-142 to 348	139%

*CoR coefficient of repeatability, LoA limits of agreement, number of subjects = 35

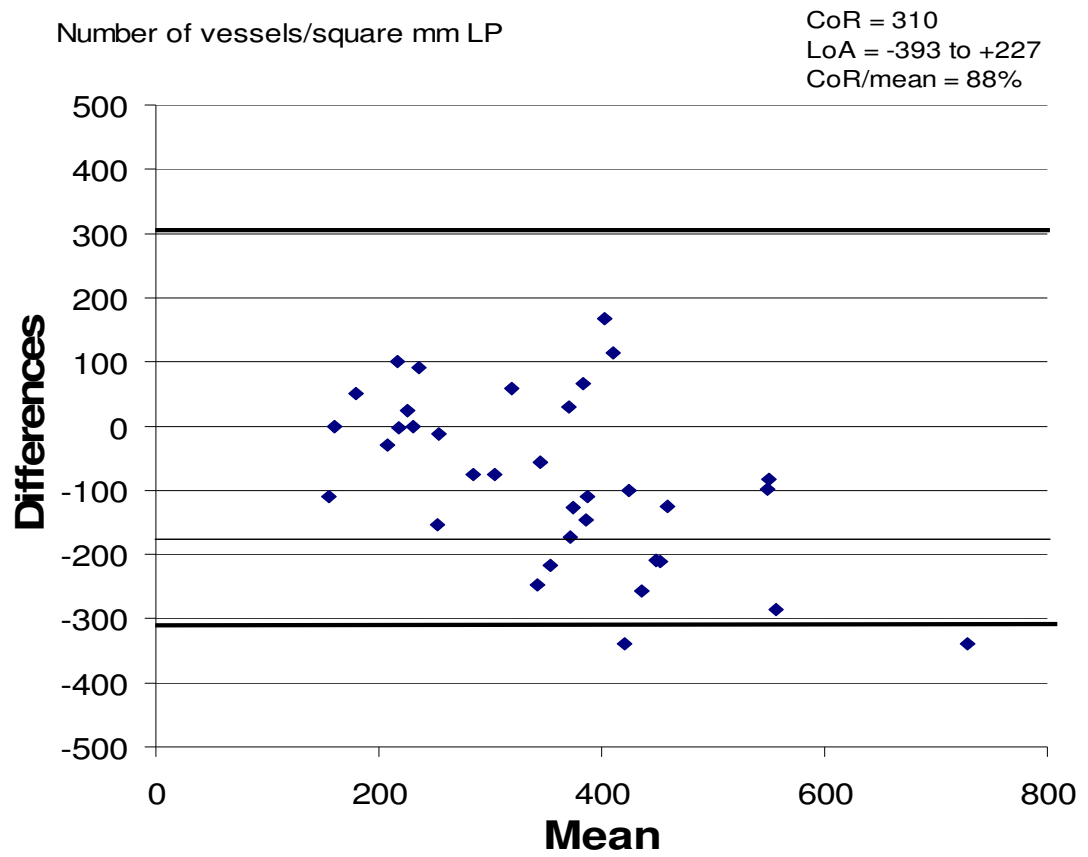


Figure 4-6. Agreement between the two methods of vessel staining for number of vessels by the Bland and Altman method.

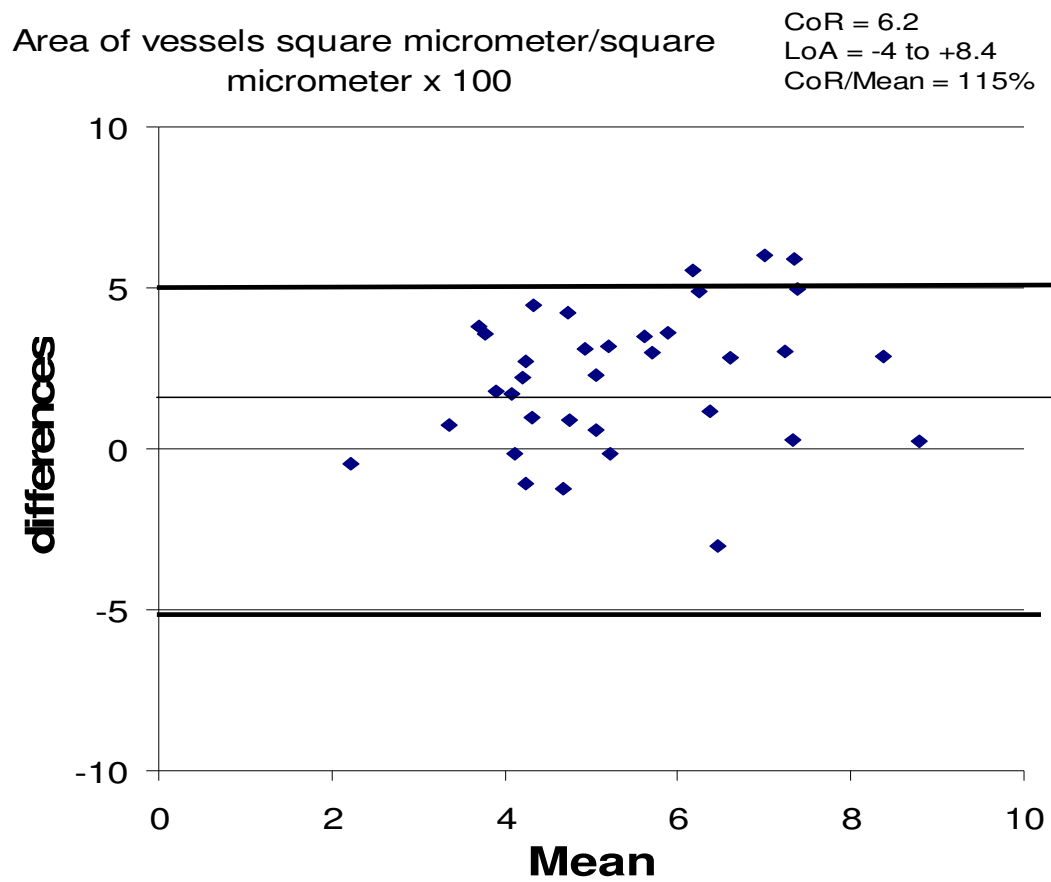


Figure 4-7. Agreement between the two methods of vessel staining for area of vessels by the Bland and Altman method.

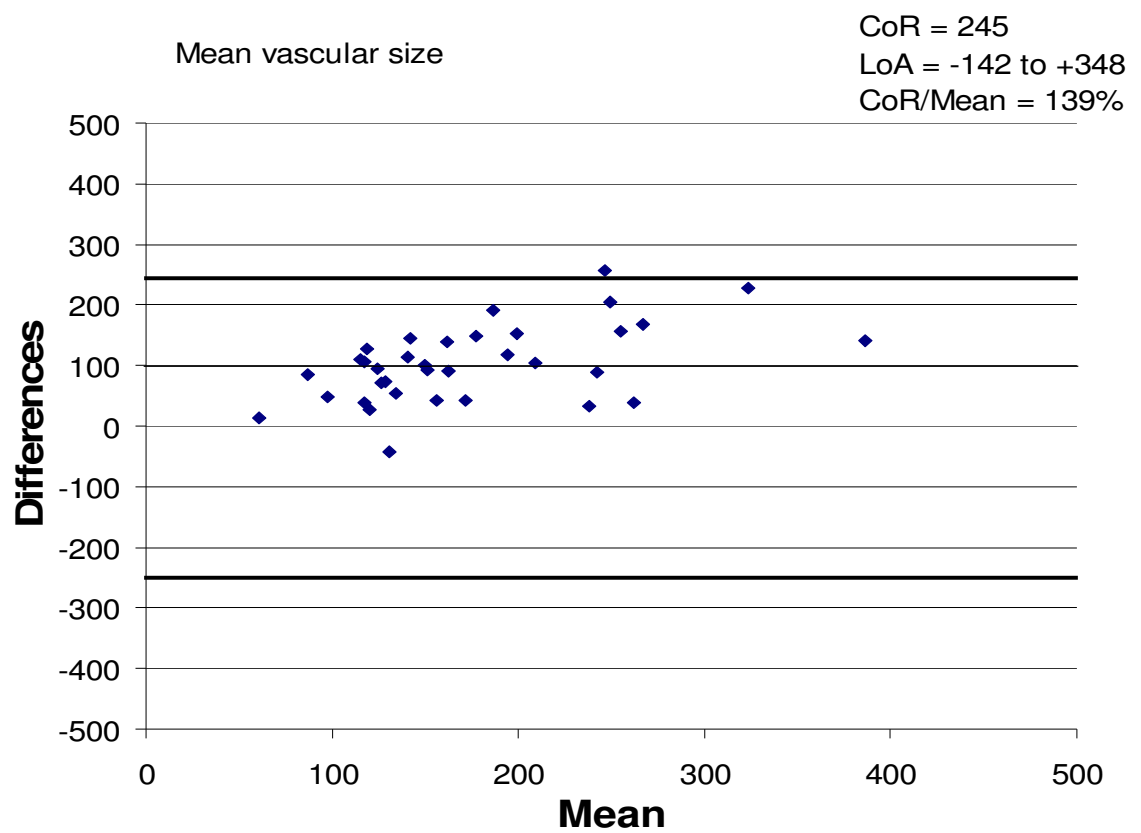


Figure 4-8. Agreement between the two methods of vessel staining for mean vascular size by the Bland and Altman method.

Table 4-3. Collagen IV versus Factor VIII in COPD and H-N together*

	Collagen IV	Factor VIII	P [†]
Number of vessels/mm ² LP	308 (100-559)	356 (154-898)	0.05 (NS)
Area of vessels ($\mu\text{m}^2/\mu\text{m}^2$ LP) x 100	6.5 (2.0-10.3)	4 (1.8-8.7)	<0.001
Mean vascular size $\mu\text{m}^2/\text{number}$	200 (67-457)	106 (45-316)	<0.001

*Number of subjects = 35, numbers are median (range) [†] Mann-Whitney test, NS = not significant

Table 4-4. Collagen IV versus Factor VIII in COPD*

	Collagen IV	Factor VIII	P [†]
Number of vessels/mm ² LP	266 (100-508)	346 (154-598)	0.15 (NS)
Area of vessels ($\mu\text{m}^2/\mu\text{m}^2$ LP) x 100	6.0 (2.0-10.3)	4.1 (1.8-8.0)	<0.001
Mean vascular size $\mu\text{m}^2/\text{number}$	204 (110-457)	112 (60-316)	<0.001

*number of subjects = 28, numbers are median (range) [†] Mann-Whitney test, NS = not significant

Table 4-5. Collagen IV versus Factor VIII in H-N*

	Collagen IV	Factor VIII	P [†]
Number of vessels/mm ² LP	349 (311-559)	474 (291-898)	0.14 (NS)
Area of vessels ($\mu\text{m}^2/\mu\text{m}^2$ LP) x 100	6.8 (3.7-8.9)	3.9 (2.6-8.7)	<0.05
Mean vascular size $\mu\text{m}^2/\text{number}$	183 (67-286)	70 (45-198)	<0.05

*number of subjects = 7, numbers are median (range)

[†] Mann-Whitney test, NS = not significant

Table 4-6. COPD versus H-N with Collagen IV antibody staining of vessels*

Groups (number)	H-N (7)	COPD (28)	P [†]
Number of vessels/mm ² LP	348 (311-559)	266 (100-508)	<0.05
Area of vessels $\mu\text{m}^2/\mu\text{m}^2$ of LP x 100	6.8 (3.7-8.9)	6 (2-10.3)	0.7 (NS)
Mean vascular size $\mu\text{m}^2/\text{number}$	183 (67-286)	204 (110-457)	0.5 (NS)

*Numbers are median (range)

[†] Mann-Whitney test, NS = not significant

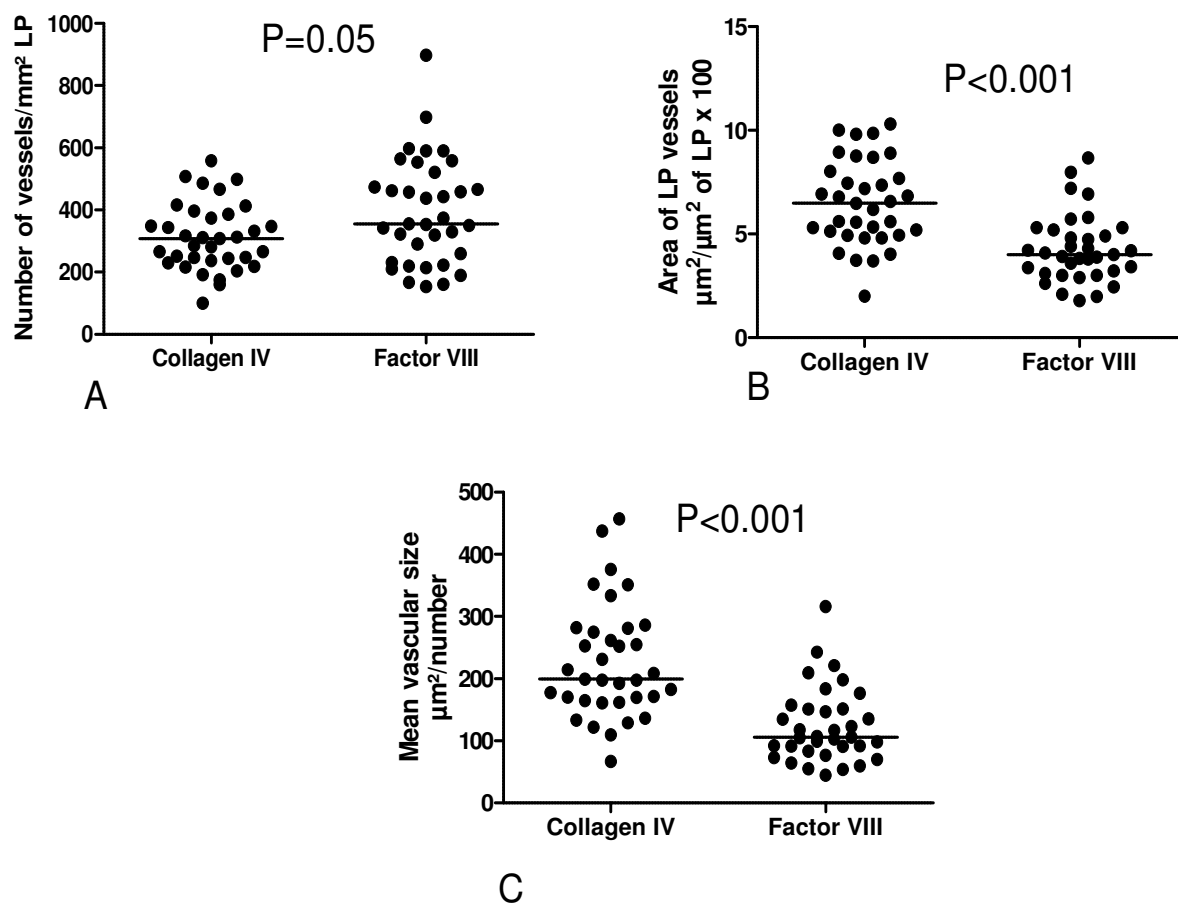


Figure 4-9. *Collagen IV versus factor VIII in COPD and H-N groups together.* Number of subjects = 35. P is calculated by the Mann-Whitney non-parametric test.

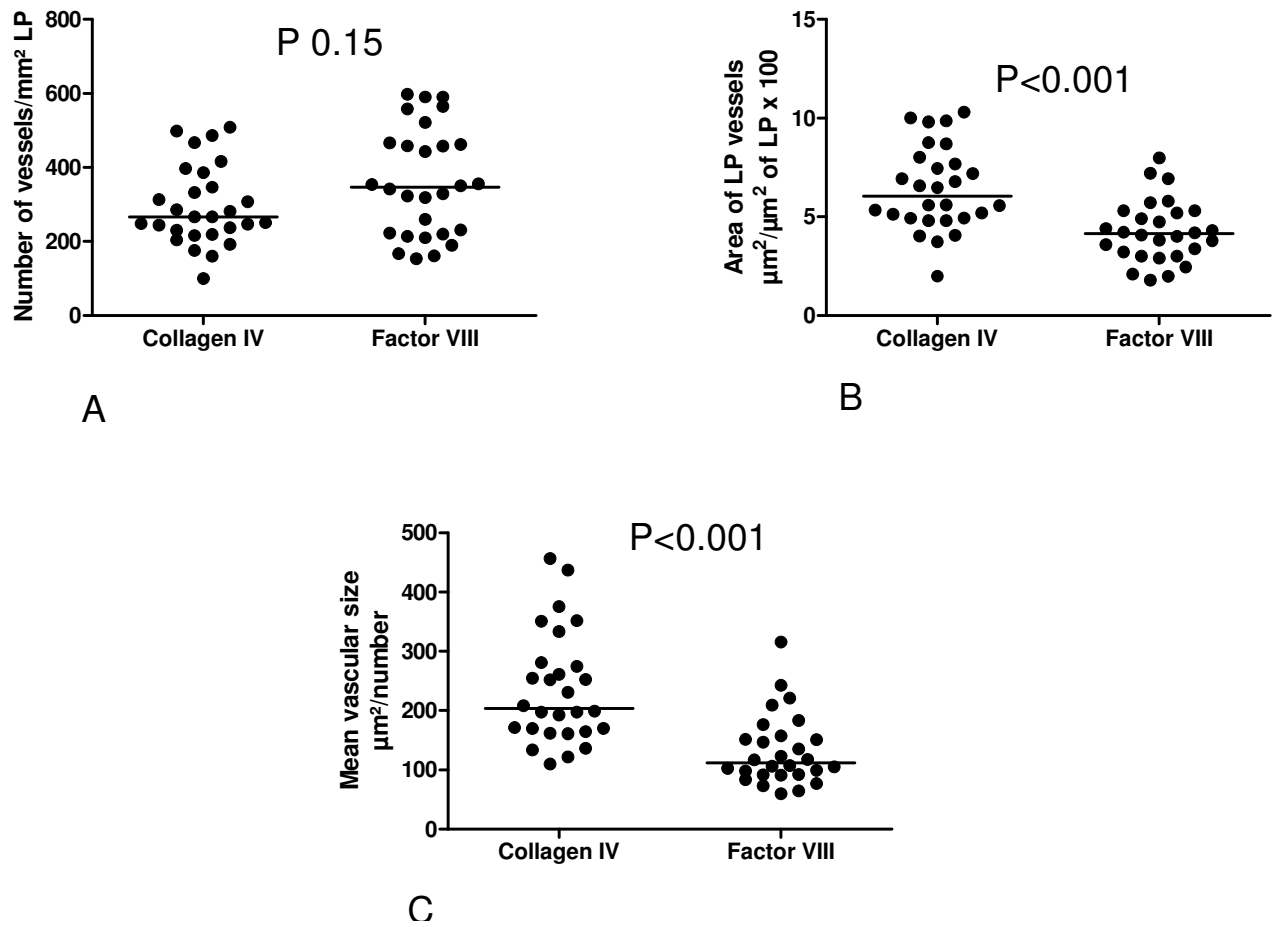


Figure 4-10. *Collagen IV versus factor VIII in the COPD group.* Number of subjects = 28. P is calculated by the Mann-Whitney non-parametric test.

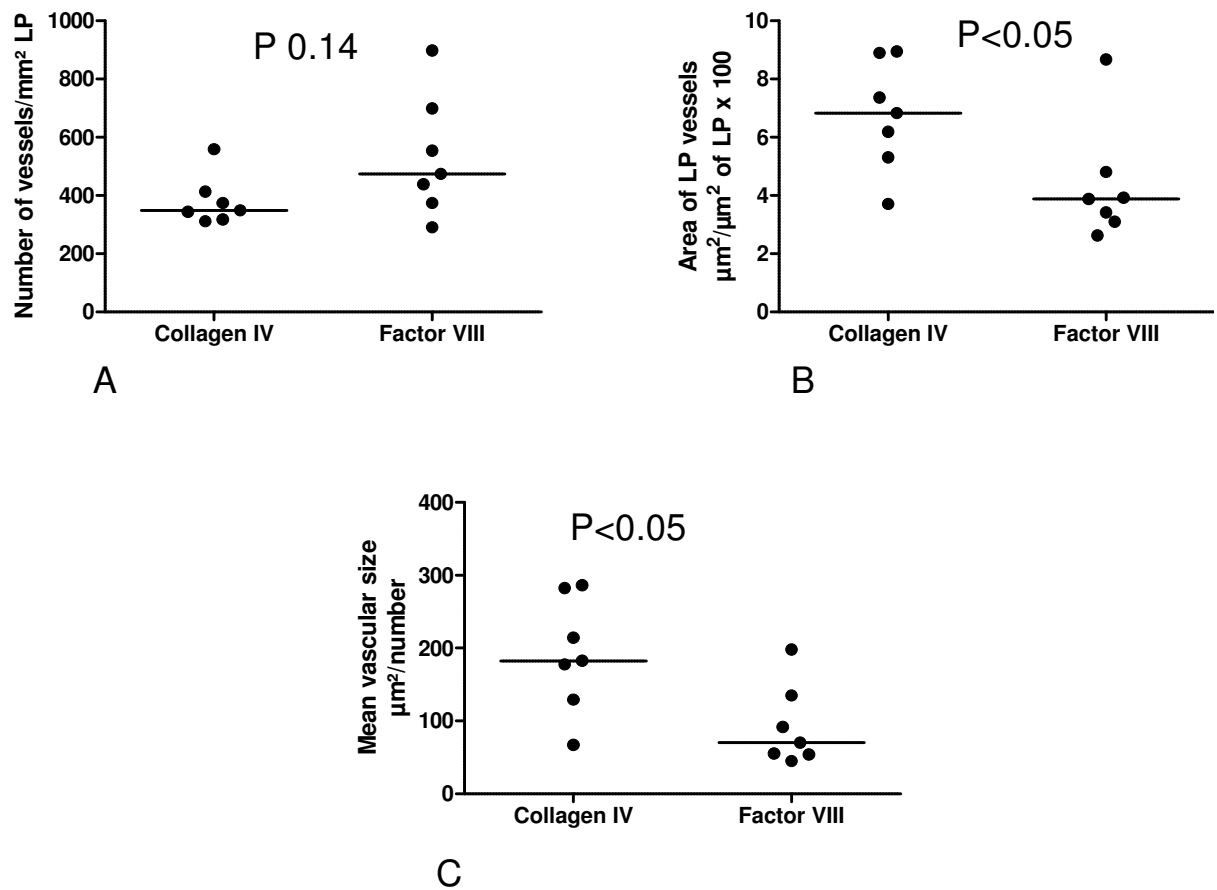


Figure 4-11. *Collagen IV versus factor VIII in the H-N group.* Number of subjects = 7. P is calculated by the Mann-Whitney non-parametric test.

Table 4-7. COPD versus H-N with Factor VIII antibody staining of vessels*

Groups (number)	H-N (7)	COPD (28)	P
Number of vessels/mm ² LP	474 (291-898)	346 (154-598)	<0.03 [¶]
Area of vessels $\mu\text{m}^2/\mu\text{m}^2$ of LP x 100	3.9 (2.6-8.7)	4.1 (1.8-8.0)	0.7 (NS) [†]
Mean vascular size $\mu\text{m}^2/\text{number}$	70 (45-198)	112 (60-316)	<0.05 [†]

*Numbers are median (range)

[†] Mann-Whitney test, NS = not significant, [¶]P value for log transformed data tested with Student's t-test

Table 4-8. Subgroups of COPD compared to H-N with Factor VIII antibody vessel staining

Groups (number)	H-N (7)	S-COPD (15)	P*	ES-COPD (13)	P* [†]
Number of vessels/mm ² LP	474 (291-898)	329 (154-591)	<0.05	350 (167-598)	0.06 (NS)

* Compared to H-N, Student's t-test, tested on log transformed data, [†]NS = Not significant

transformed data $p < 0.05$], but not between ES-COPD and H-N [median (range) 350 (167-598) for ES-COPD vs. 474 (291-898), Student's t-test for log transformed data $p = 0.06$ (not significant)] (Table 4-8). The COPD group also had higher MVS compared to controls with Factor VIII [median (range) 112 (60-316) vs. 70 (45-198), $p < 0.05$] (Table 4-7).

Discussion:

This study revealed that Collagen IV and Factor VIII monoclonal antibody staining outline somewhat different structures, and gives a different profile for the airway lamina propria vasculature.

Linear correlations between the two methods of vessel staining (Figures 4-3 to 4-5) and Bland and Altman plots (Figures 4-6 to 4-8) indicate an increasing number of vessels staining with Factor VIII antibody than with Collagen IV antibody (values on the Y axis in Figure 4-6 are calculated as the number of vessels counted with Collagen IV staining minus the number of vessels counted with Factor VIII staining). When the number of vessels was relatively few, around 150-350 per mm^2 of the LP, Collagen IV antibody and Factor VIII antibody were better correlated. As the number of vessels increased, many vessels which were detected with Factor VIII antibody were missed with Collagen IV antibody (Figures 4-3 and 4-6). By showing the difference between the two methods of vessel quantification, Figure 4-9A supports this finding ($p = 0.05$).

Figure 6-4 shows that the two methods of vessel staining do not correlate well in measuring the area of vessels in the LP. Figure 4-7 supports this finding. The two methods show narrow differences around vascular area between 3-6 μm^2 but wide differences for higher levels of vascular area. Figures 4-9B to 4-11B illustrate that Collagen IV staining revealed significantly greater vascular area than did Factor VIII antibody staining.

Figure 4-5 reveals good correlation between the two methods of staining for MVS, but Figure 4-8 shows that the agreement between the two methods was best when vessels were relatively smaller. As MVS increases there are increasing differences between the two methods, with Collagen IV showing higher MVS than Factor VIII (values in Y axis are calculated as MVS measured with Collagen IV minus MVS measured with Factor VIII). Figures 4-9C to 4-11C support that there being a significant difference between Collagen IV antibody and Factor VIII antibody staining for MVS.

To summarise all the above findings: this study shows that Factor VIII antibody is more sensitive in detection of smaller vessels and stains more of them. Small vessels are likely to be newer vessels which have not yet developed their BM, so are more easily detected with Factor VIII than with Collagen IV. Therefore, in conditions where we expect active new vessel formation, such as malignancies, Factor VIII antibody may detect new vessels better than Collagen IV antibody. In contrast, vessels which are larger and probably more mature, are more effectively detected when stained with Collagen IV antibody rather than with Factor VIII antibody.

It has previously been shown that vessel markers can have different sensitivity and specificity in detecting vessels in normal versus abnormal conditions. For example, factors like genetic diversity of endothelial cells, hypoxemia, age and shearing stress have effects on the expression of Factor VIII protein (Pusztaszeri et al., 2006) (Muller, Hermanns et al., 2002; Muller, Skrzynski, Nesslinger, Skipka, & Muller, 2002). The sensitivity and specificity of these markers are also related to the size of vessels (Bettelheim et al., 1984; Fina et al., 1990). But this type of differentiation has not previously been attempted with airway wall samples.

I would propose that the greater MVS with Collagen IV compared to Factor VIII immunostaining indicates that Collagen IV antibody is unable to delineate small and newer vessels that have not matured sufficiently to fully develop their BM; and Factor VIII antibody probably can not detect larger vessels, at least as well. Indeed, it has

previously been shown that staining for Factor VIII cannot detect larger sized vessels as accurately as smaller ones in invasive breast cancer (Bettelheim et al., 1984).

An *in vivo* study on mice showed that new vessels in airways formed under angiogenic stimulation of VEGF had detectable pericytes and BM on day 7. When the newly formed vessels were deprived of VEGF, firstly the flow of blood stopped, followed by death and fragmentation of endothelial cells and then apoptosis of pericytes. However, a basement membrane sleeve from the whole structure remained for some time (Baluk et al., 2004). This just goes to emphasise that different markers will work better or worse depending on the age, maturity and growth factor environment of vessels, and that these are not static conditions.

The difference between these two quantification methods could also be partly the result of confounding by structures other than vascular tissue being stained by these markers. For example, Factor VIII antibody has been reported to stain lymphatic endothelial cells as well as blood vessel tissue (Horak et al., 1992; Parums et al., 1990).

In addition to differences between immunohistochemical methods, this study demonstrated differences between COPD and H-N groups using both vessel markers. With both Collagen IV and Factor VIII antibodies, the COPD group had fewer vessels in the LP. In the COPD group MVS was greater when compared with H-N using Factor VIII. These two differences suggest that in COPD the LP has fewer but larger and more “mature”, i.e. older, vessels but the precision of showing these changes differed between the markers used. This suggests less active angiogenesis in the LP in COPD, which fits with my suggestive overall lower vascularisation within the LP in COPD.

Thus, comparison of vessels stained with Factor VIII antibody confirmed our results in the cross-sectional study (Chapter Five) with Collagen IV antibody which revealed hypovascularity of the LP.

As smaller (probably “newer”) vessels are better detected with Factor VIII antibody and larger (probably “mature”) vessels are better detected with Collagen IV antibody, it could be advantageous to stain tissues with both these vessel markers in states that are characterised by new vessel formation and active angiogenesis, like malignancies and inflammatory diseases. This could also be useful in evaluating the effects of treatments on vascular regression; such as my longitudinal study which assessed the effects of ICS on vessels in the airways of COPD subjects.

We conclude that in BB from normal controls and COPD, antibodies against vessel markers Factor VIII and Collagen IV do not uniformly detect all vessels of different sizes and can potentially be confounded by detecting structures other than vessel endothelium.

Chapter Five

Airway Remodelling in COPD: Cross-Sectional Study

Abstract:

Background: As previously discussed in Chapters One and Two, little is known about airway remodelling in bronchial biopsies (BB) from smokers and patients with chronic obstructive pulmonary disease (COPD). As discussed in Chapter One, I conducted an initial, pilot study comparing BB from COPD patients with nonsmoking controls which suggested the presence of reticular basement membrane (Rbm) fragmentation and altered vessel distribution in COPD.

Methods: We designed a cross-sectional study to determine whether Rbm fragmentation and altered vessel distribution in BB were specific for COPD or were only smoking effects. BB from 19 current smokers and 14 ex-smokers with mild to moderate COPD were stained and compared with those of 15 current smokers with normal lung function and 17 healthy and nonsmoking subjects.

Results: Thickness of the Rbm was quite variable both within and between individuals with COPD but was not significantly different between groups. The Rbm showed fragmentation and splitting in both current smoking groups and also ex-smokers with COPD when compared with healthy nonsmokers ($P < 0.02$); smoking and COPD seemed

descriptively to have additive effects. There were more vessels in the Rbm and fewer vessels in the lamina propria in current smokers compared to healthy nonsmokers ($p < 0.05$). The number of vessels which stained for vascular endothelial growth factor (VEGF) in the Rbm was higher in both current smoker groups and ex-smokers with COPD compared to healthy nonsmokers ($p < 0.005$). The percentage of vessels staining for VEGF was also increased in the same groups. In current smokers with COPD, both total number of vessels and the percentage of vessels staining with VEGF correlated with FEV1% predicted ($r = 0.61$, $p < 0.02$ and $r = 0.61$, $p < 0.01$ respectively).

Conclusions: Airway remodelling in smokers and patients with mild to moderate COPD is associated with fragmentation of the Rbm and altered distribution of vessels in the airway wall. Rbm fragmentation seems nonreversible despite smokers having quit. These characteristics may have potential physiological consequences.

Introduction:

COPD and asthma are common inflammatory airway diseases. There have been many reports about airway remodelling in asthma during the last two decades. Changes in Rbm morphology and airway wall vascularity and the response of these changes to anti-inflammatory treatment in asthmatics have been the subject of many investigations (Bryce N. Feltis et al., 2006; Jeffery, 2001; Knox et al., 2005) (B. N. Feltis et al., 2007; Olivieri et al., 1997). As I have said in previous chapters, compared to the work done on asthma, little is known about airway remodelling in airways, and large airways in particular, and its relation to physiology in COPD (Bergeron & Boulet, 2006; James & Wenzel, 2007). I should emphasise that most data for COPD are from studies on lung parenchyma and small airways in surgically resected specimens (Jeffery, 2001). As far as we know, only two groups of investigators used BB to study vascularity changes in large airways in COPD (Calabrese et al., 2006; Zanini et al., 2009).

As was previously explained in Chapter One, our preliminary study showed changes in the Rbm and vessel distribution in current smokers in COPD. On the basis of these findings we hypothesised that Rbm splitting and vascularisation changes are specific features of COPD. We also aimed to investigate VEGF and TGF- β status in our larger cross-sectional study. In this comprehensive study we have compared BB from healthy, nonsmoking subjects (H-N), current smokers with COPD (S-COPD) and ex-smokers with COPD (ES-COPD). At this stage we have not investigated ex-smoker physiologically normal individuals. However, to further discriminate between smoking and disease effects we included BB from current smokers with normal lung function (S-N) as a highly relevant control group.

Hypotheses:

1. Rbm splitting and vascular changes are specific features of COPD.
2. These changes may be related to VEGF activity.
3. These changes may be related to TGF- β 1 activity.

Material and methods:

See Chapters One and Three for details on subject recruitment, tissue sampling, processing and measurements.

Results:

Sixty-three from sixty-five participating subjects had sufficient tissue for VEGF staining quantification, and fifty-nine subjects for Collagen IV staining studies. Only forty five samples were acceptable for TGF- β examination.

Demographics are presented in Table 5-1. COPD groups were significantly older than subjects who had normal lung function, and as expected by our inclusion criteria, they had significantly lower FEV1% predicted and FER. We have looked at whether our data had age-related dimensions. Univariable correlation tests revealed no relationship between age and any of remodelling parameters in each one of our study groups except for Rbm splitting in the ES-COPD group and Rbm vascular area in the H-N group (Table 5-2). However, multivariable analysis (Table 5-3) showed that pack-year smoking history and not age was the predictor of splitting in the ES-COPD group [correlation coefficient (95% CI) for age +1.98 (-1.19 to +5.160), $p = 0.2$; and for pack-year smoking history +0.46 (+0.03 to +0.90), $p < 0.05$].

Rbm morphology (Table 5-4 and Figures 5-1 & 5-2): Thickness of the Rbm (μm) was variable in COPD subjects and was not significantly different between the four groups [median (range) for H-N, S-N, S-COPD and ES-COPD 5.2 (2.3-9.6), 6.7 (3.4-14.0), 6.4 (2.7-17.0) and 5.7 (1.0-7.5) respectively, non-parametric ANOVA (Kruskal-Wallis) test, $p = 0.13$]. The length of Rbm splits ($\mu\text{m}/\mu\text{m}$ Rbm $\times 100$) as an index of fragmentation (see also Figure 1-3 Chapter One) in S-N and both COPD groups was significantly greater than normal controls [median (range) for S-N, S-COPD and ES-COPD 10 (1-90), 20 (0-68) and 12 (0-110) respectively vs. H-N 5 (0-20), non-parametric ANOVA test, $p < 0.02$]. Splits were not significantly different between S-N, S-COPD and ES-COPD groups, but did appear especially marked in S-COPD.

Table 5-1. Demographics

Groups* (numbers)	H-N (n=17)	S-N (n=15)	S-COPD (n=19)	ES-COPD (n=14)	P value
Age [†] years	54 (20-68)	46 (30-65)	61 (46-78)	61 (53-69)	<0.01
Female/male	6/11	4/11	8/11	5/9	0.2 (NS) ^p
Pack-years of smoking [†]	0	35 (11-57)	45 (18-82)	55 (18-151)	<0.01
FEV1%pred icted ^{†‡}	119 (114-124)	100 (78-125)	83 (55-102)	83 (55-105)	<0.01
FER ^{†‡}	82 (71-88)	78 (70-96)	59 (46-68)	57 (38-68)	<0.01
DLCO% predicted [†] ml/min/mm Hg	-	77 (58-105)	67 (48-83)	64 (45-74)	<0.01

* H-N: healthy and nonsmoker, S-N: smokers with normal lung function, S-COPD and ES-COPD: smokers and ex-smokers with COPD

[†]Median (range), [‡] post-bronchodilator values, ^pChi-square test, NS = not significant

Table 5-2. Correlation between age and remodelling parameters

Remodelling parameter	H-N r (r ²)	S-N r (r ²)	S-COPD r (r ²)	ES-COPD r (r ²)
Rbm splitting	-0.4 (0.16) P = 0.2	0.1 (0.01) P = 0.7	0.2 (0.04) P = 0.4	0.6 (0.36) P<0.05
No. of Rbm vessels	0.5 (0.25) P = 0.1	0.3 (0.09) P = 0.3	0.2 (0.04) P = 0.5	0.2 (0.04) P = 0.4
Area of Rbm vessels	0.6 (0.36) P<0.05	0.5 (0.25) P = 0.7	-0.2 (0.04) P = 0.5	0.4 (0.16) P = 0.2
No. of LP vessels	0.1(0.01) P = 0.8	0.03 (0.00) P = 0.9	0.2 (0.04) P = 0.4	-0.4 (0.16) P = 0.1
Area of LP vessels	0.2 (0.04) P = 0.5	0.4 (0.16) P = 0.1	-0.1 (0.01) P = 0.7	0.1 (0.01) P = 0.6
No. of VEGF positive vessels	-0.2 (0.04) P = 0.5	-0.4 (0.16) P = 0.2	-0.05 (0.00) P = 0.9	0.2 (0.04) P = 0.6
Area of VEGF positive vessels	-0.2 (0.04) P = 0.5	-0.3 (0.09) P = 0.3	-0.01 (0.00) P = 0.9	-0.1 (0.01) P = 0.8
No. of TGF- β positive vessels	0.02 (0.00) P = 1.0	-0.02 (0.00) P = 1.0	0.05 (0.00) P = 0.9	0.5 (0.25) P = 0.1
Area of TGF- β positive vessels	0.02 (0.00) P = 1.0	-0.2 (0.04) P = 0.6	-0.03 (0.00) P = 0.4	0.4 (0.16) P = 0.3

* Spearman correlation coefficient

Table 5-3. Multivariable analysis for correlation between age and pack-year smoking history with Rbm splitting in the ES-COPD group

	Coefficient	95% CI	P*
Age	+1.98	-1.19 to +5.16	0.2 (NS)
Pack-year smoking	+0.46	+0.03 to +0.90	<0.05

*NS = Not significant

Vessels in the Rbm (Table 5-4 and Figure 5-3, see also Figure 1-5, Chapter One): Both the number of vessels in the Rbm/mm Rbm [median (range) for S-N and S-COPD 7.9 (3.0-23.0) and 10.0 (1.6-23.0) respectively vs. H-N 4.4 (0.0-26.0), $p < 0.05$ for both] and area of Rbm vessels $\mu\text{m}^2/\text{mm Rbm}$ [median (range) for S-N and S-COPD vs. H-N 1108 (507-5064) and 953 (115-2456) vs. 462 (0-3263), $p < 0.01$ and $p < 0.05$ respectively] were significantly different in current smoking groups compared to H-N [non-parametric ANOVA (Kruskal-Wallis) test, $p < 0.05$ for number of vessels and $p < 0.02$ for area of vessels] while the ES-COPD group was essentially normal [median (range) for number of vessels 4.7 (2.5-21.0), $p = 0.3$ compared to controls and for area of vessels 460 (105-1503), $p = 0.6$ compared to controls]. The area of vessels $\mu\text{m}^2/\text{mm Rbm}$ was significantly higher in S-N than in ES-COPD ($p < 0.02$) and when both current smoking groups taken together were compared with ES-COPD [median (range) 1011 (115-5064) vs. 460 (105-1503), $p < 0.02$].

Vessels in the LP (Table 5-5 and Figure 5-4): The density of vessels in the LP (number of vessels/ mm^2 LP) was significantly lower in the two currently smoking groups compared to H-N (ANOVA test, $p < 0.05$), [mean (SD) for S-N and S-COPD 216 (93) and 229 (125) respectively vs. H-N 353 (108) respectively, $p < 0.01$ for both], while ES-COPD had normal values [mean (SD) 323 (134), $p = 0.6$ compared to controls]. S-N and both current smoking groups taken together had significantly lower vascular density (number/ mm^2 LP) [mean (SD) 216 (93) and 223 (110)] than ES-COPD [mean (SD) 323 (134), $p < 0.01$ and $p < 0.02$ respectively]. The area of vessels in the LP ($\mu\text{m}^2/\mu\text{m}^2$ of LP \times 100) was significantly lower in S-COPD compared to H-N [mean (SD) 4.7 (1.9) vs. 7.0 (2.8), $p < 0.02$].

VEGF (Table 5-6 and Figure 5-5): The number/mm Rbm [median (range) for H-N, S-N, S-COPD and ES-COPD 0.0 (0.0-9.0), 0.7 (0.0-3.3), 1.4 (0.0-6.9) and 1.0 (0.0-5.4) respectively, non-parametric ANOVA (Kruskal-Wallis) test, $p < 0.005$] and area

Table 5-4. Rbm changes[†]

Groups (numbers)	H-N (n=13)	S-N (n=15)	S-COPD (n=18)	ES-COPD (n=13)	P value*
Rbm thickness μm	5.2 (2.3-9.6)	6.7 (3.4-14.0)	6.4 (2.7-17.0)	5.7 (1.0-7.5)	0.13 (NS)
Rbm splitting $\mu\text{m}/\mu\text{m}$ Rbm x 100	5 (0-20)	10 (1-90)	20 (0-68)	12 (0-110)	<0.02
No. of Rbm vessels/mm Rbm	4.4 (0.0-26.0)	7.9 (3.0-23.0)	10.0 (1.6-23.0)	4.7 (2.5-21.0)	<0.05
Area of Rbm vessels $\mu\text{m}^2/\text{mm}$ Rbm	462 (0-3263)	1108 (507-5064)	953 (115-2456)	460 (105-1503)	<0.02

*Kruskal-Wallis test, NS = not significant, [†] All values are median (range)

Table 5-5. Vascular changes in the LP[†]

Groups (numbers)	H-N (n=13)	S-N (n=15)	S-COPD (n=18)	ES-COPD (n=13)	P value*
No. of LP vessels/ mm^2 LP	353 (108)	216 (93)	229 (125)	323 (134)	<0.05
Area of LP vessels $\mu\text{m}^2/\mu\text{m}^2$ of LP x 100	7.0 (2.8)	5.5 (2.7)	4.7 (1.9)	6.4 (2.6)	<0.05

*ANOVA comparing three groups of both current smokers and H-N

[†] All values are mean (SD)

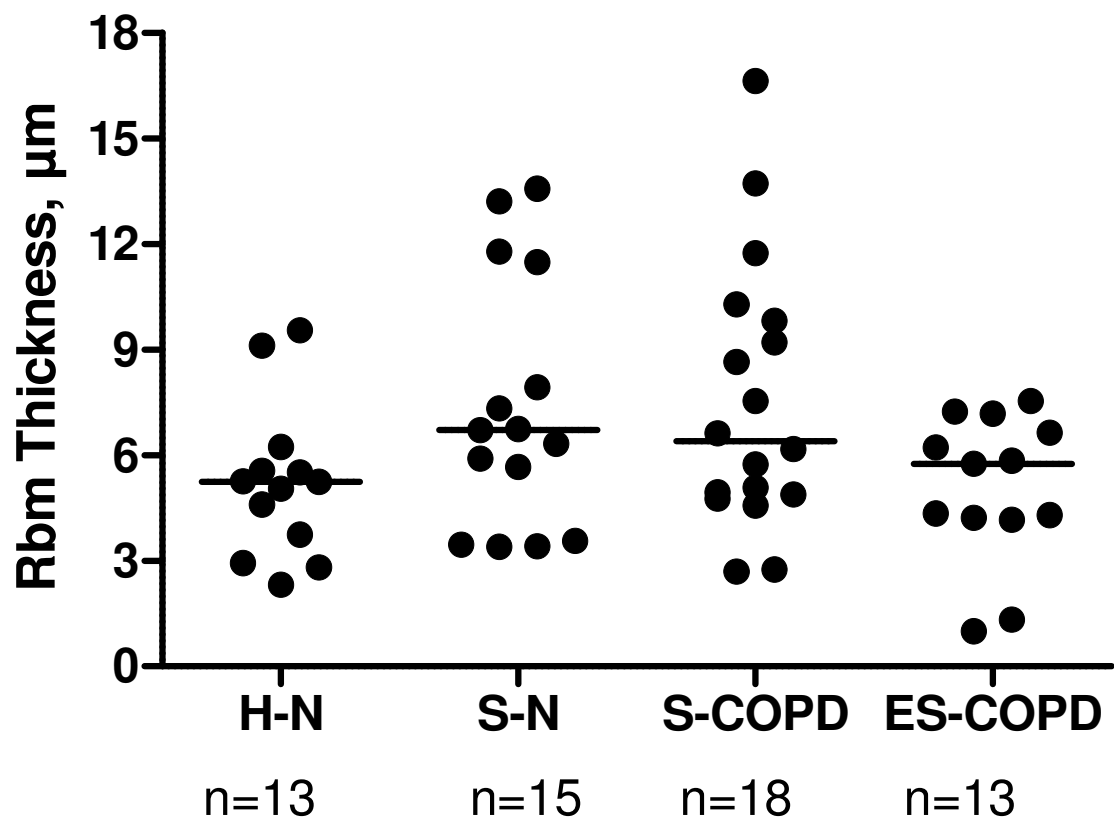


Figure 5-1. *Rbm thickness.* $P = 0.13$ (non-significant).

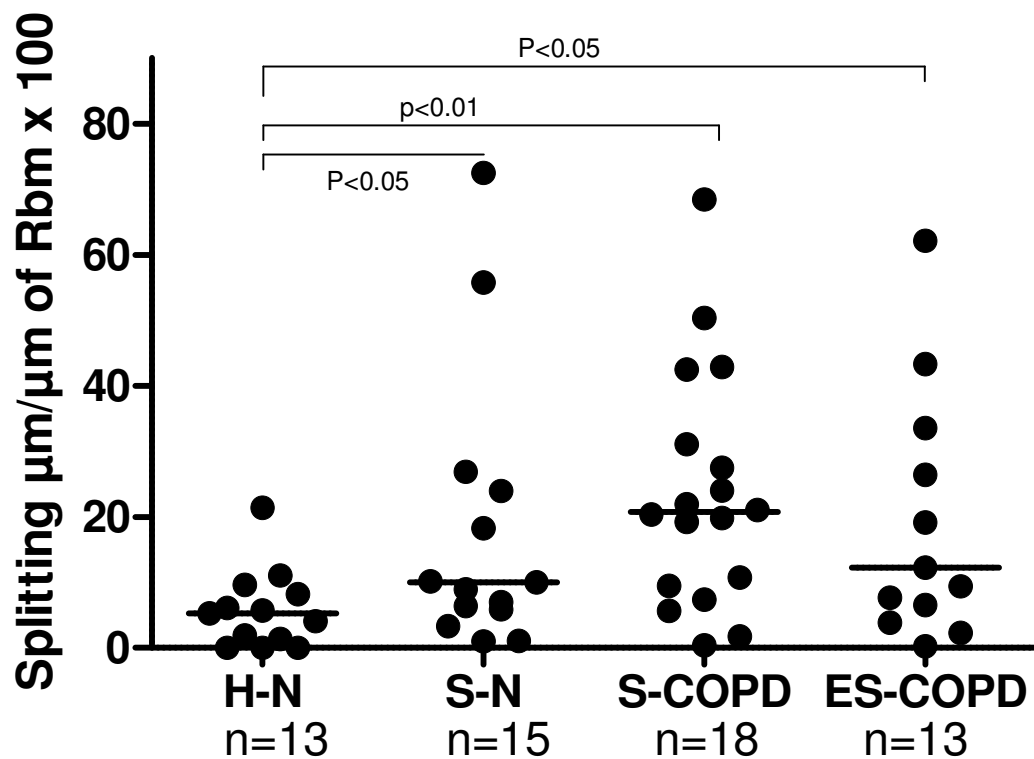


Figure 5-2. *Rbm splitting.* One dot point in the S-N group and one dot point in the ES-COPD group were greater than 80 and thus are not shown in this graph.

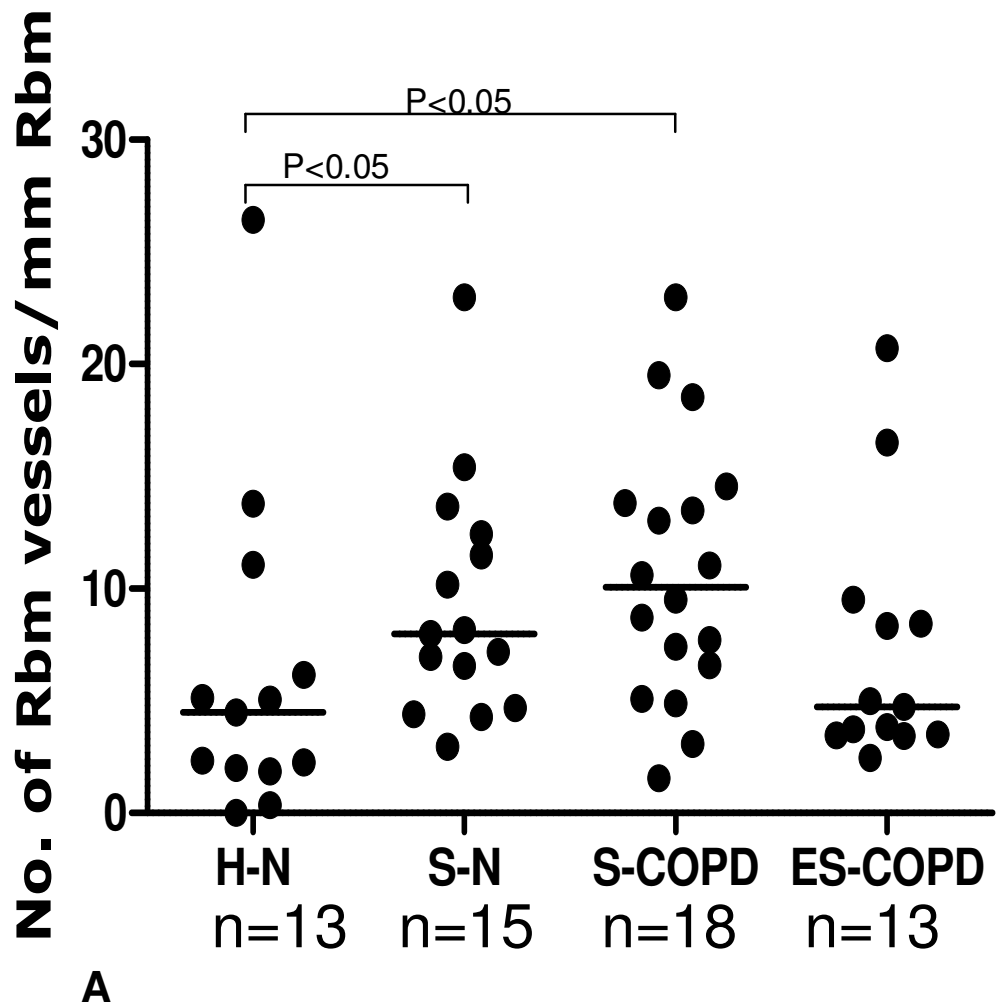


Figure 5-3A. *Number of Rbm vessels.*

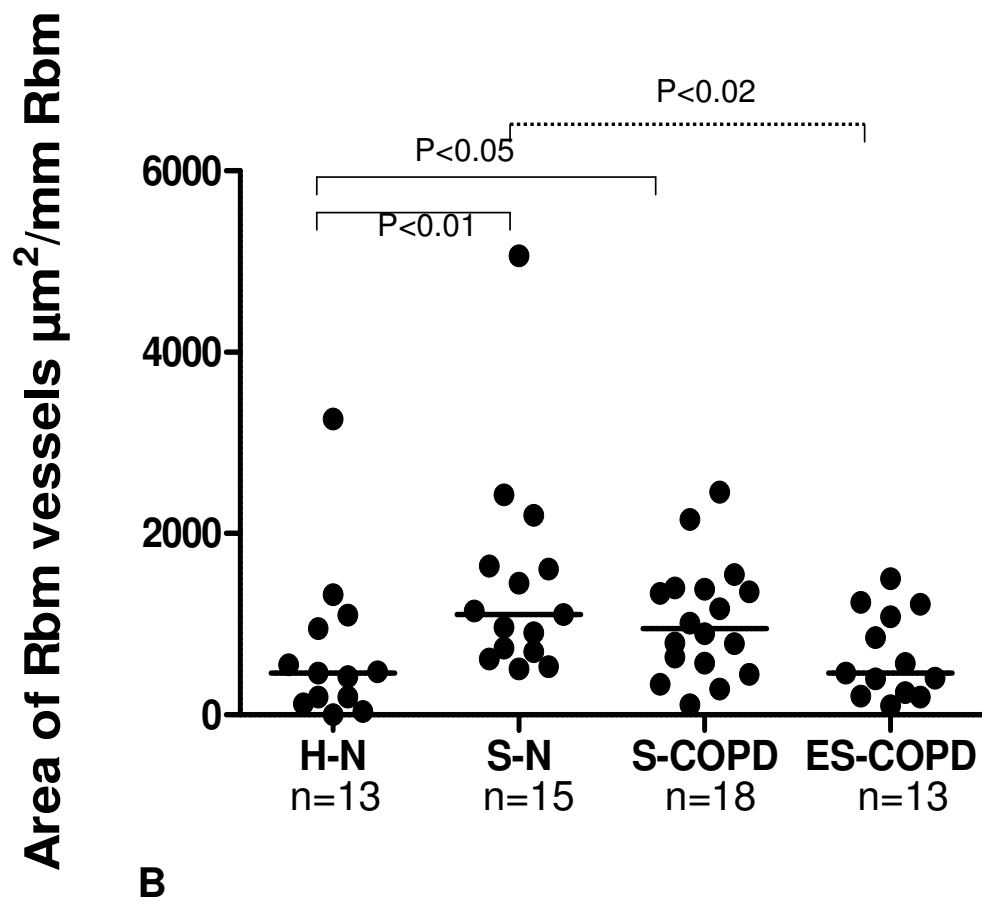


Figure 5-3B. *Area of Rbm vessels.*

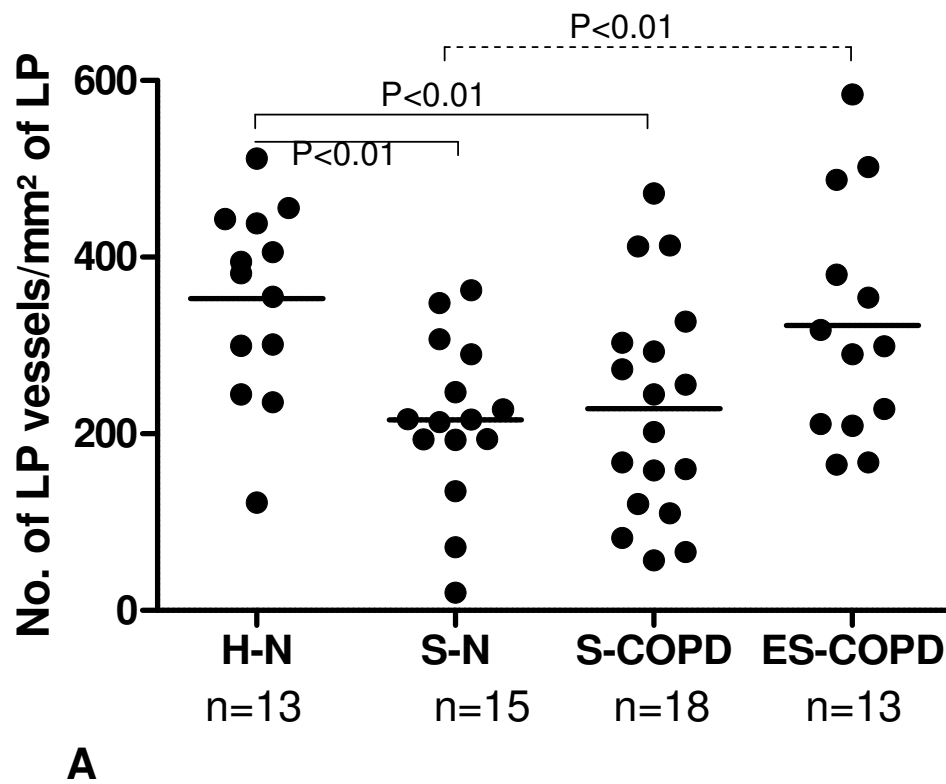


Figure 5-4A. *Number of LP vessels.*

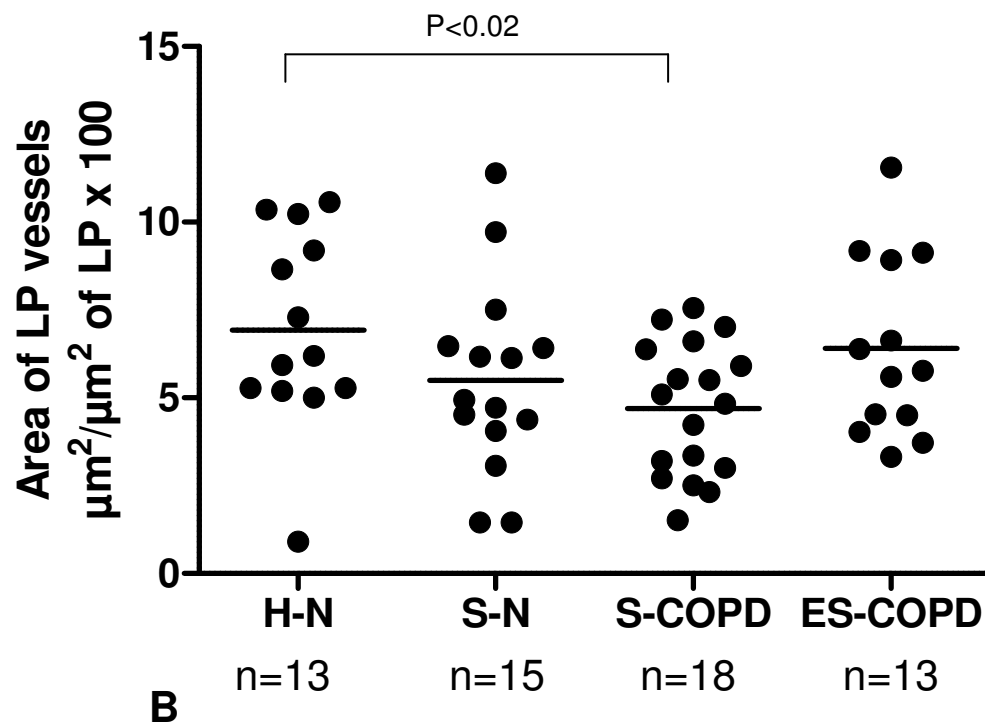


Figure 5-4B. *Area of LP vessels.*

($\mu\text{m}^2/\text{mm Rbm}$) [median (range) for H-N, S-N, S-COPD and ES-COPD 0 (0-1115), 51 (0-564), 91 (0-746) and 28 (0-610), non-parametric ANOVA (Kruskal-Wallis) test, $p<0.005$] of vessels stained for VEGF in the Rbm were significantly different between groups, the increase being most marked for the S-COPD group. Further, the percentage of vessels stained for VEGF (ratio of vessels stained for VEGF divided by total number of vessels stained with Collagen IV x 100) in the Rbm was significantly higher in S-N, S-COPD and ES-COPD compared to H-N [median (range) 9 (0-41), 21 (0-92) and 18 (0-108) vs. 0 (0-34) respectively for percent number of vessels, non-parametric ANOVA test, $p<0.01$; 4 (0-61), 12 (0-99) and 6 (0-84) vs. 0 (0-34) respectively for percent area of vessels, non-parametric ANOVA test $p<0.005$] (Figure 5-6).

TGF- β (Table 5-7 and Figure 5-7): Number of vessels stained for TGF- β in the Rbm/mm Rbm was higher in S-N, S-COPD and ES-COPD compared to normal controls [median (range) 2.5 (0.0-12.7), 3.4 (0.0-8.1) and 1.0 (0.0-6.3) vs. 0.0 (0.0-7.0), non-parametric ANOVA (Kruskal-Wallis test) $p<0.02$]. Area of vessels stained for TGF- β in the Rbm $\mu\text{m}^2/\text{mm Rbm}$ was significantly greater in S-N, S-COPD and ES-COPD compared to normal controls [median (range) 379 (0-2132), 324 (0-2882) and 155 (0-4029) vs. 0 (0-545), non-parametric ANOVA (Kruskal-Wallis test) $p<0.007$]. The percentage number of vessels stained for TGF- β (ratio of number of vessels stained for TGF- β /total number of vessels stained with Collagen IV) was also higher in S-N, S-COPD and ES-COPD compared to controls [median (range) 31 (0-121), 41 (0-311) and 22 (0-114) vs. 0 (0-26), non-parametric ANOVA test $p<0.03$]. The percentage area of vessels stained for TGF- β was greater in S-N, S-COPD and ES-COPD compared to controls [median (range) 37 (0-194), 48 (0-503) and 26 (0-324) vs. 0 (0-24), non-parametric ANOVA test $p<0.02$] (Figure 5-8).

Table 5-6. Results for VEGF[†]

Groups (numbers)	H-N (n=17)	S-N (n=15)	S-COPD (n=18)	ES-COPD (n=13)	P value*
No. of vessels stained for VEGF/mm Rbm	0.0 (0.0-9.0)	0.7 (0.0-3.3)	1.4 (0.0-6.9)	1.0 (0.0-5.4)	<0.005
Area of vessels stained for VEGF $\mu\text{m}^2/\text{mm}$ Rbm	0 (0-1115)	51 (0-564)	91 (0-746)	28 (0-610)	<0.005
Percent of No. of Rbm vessels stained for VEGF/mm Rbm x 100 [‡]	0 (0-34)	9 (0-41)	21 (0-92)	18 (0-108)	<0.01
Percent of area of Rbm vessels stained for VEGF/mm Rbm x 100 [‡]	0 (0-34)	4 (0-61)	12 (0-99)	6 (0-84)	<0.005

*Kruskal-Wallis test, [†] All values are median (range)

[‡] Calculated as number and area of vessels respectively in the Rbm stained for VEGF divided by number and area of vessels stained for collagen IV x 100.

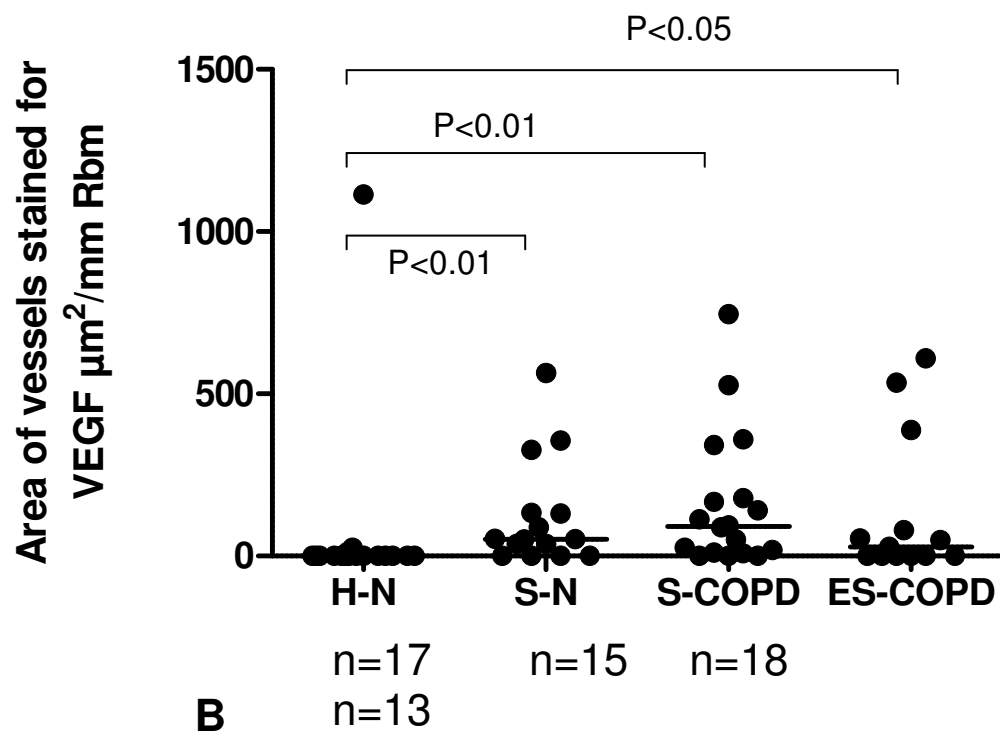


Figure 5-5B. *VEGF staining of Rbm vessels, area of vessels.*

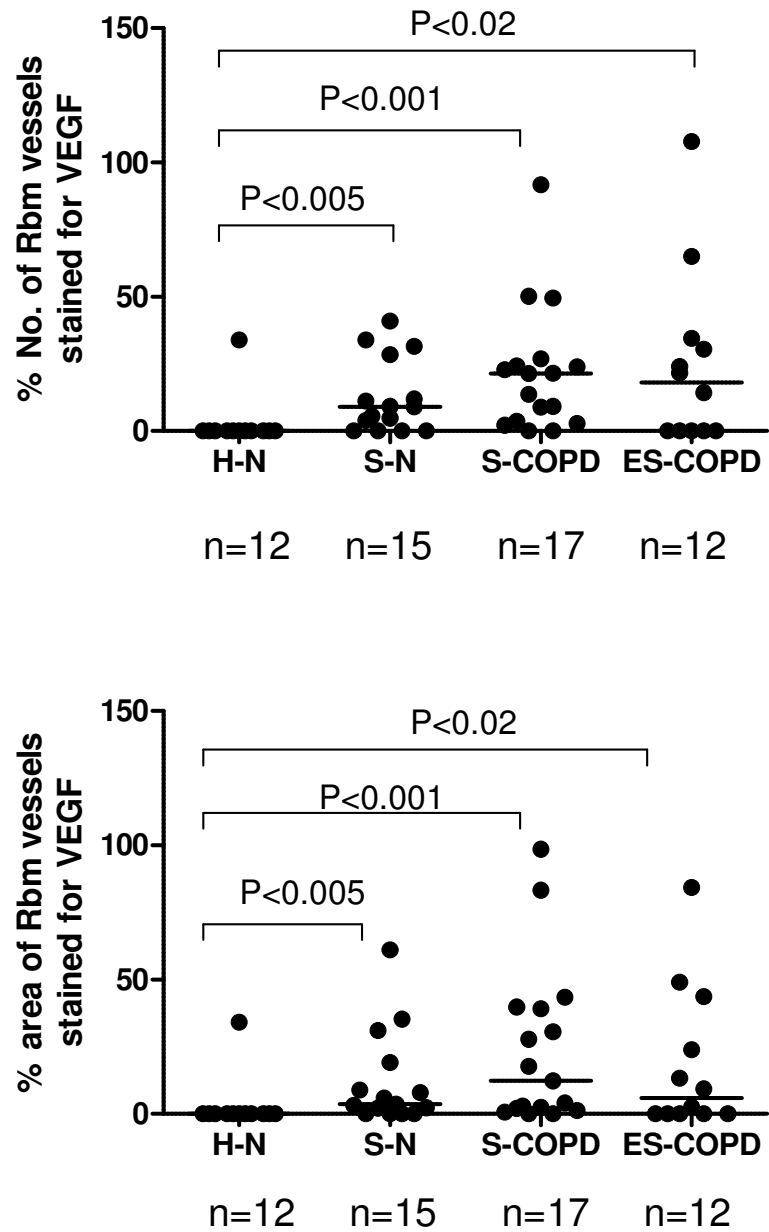


Figure 5-6. Percent of Rbm vessels stained for VEGF.

Table 5-7. Results for TGF- β 1[†]

Groups (numbers)	H-N (n=10)	S-N (n=12)	S-COPD (n=11)	ES-COPD (n=12)	P value*
No. of vessels stained for TGF- β 1 /mm Rbm	0.0 (0.0-7.0)	2.5 (0.0-12.7)	3.4 (0.0-8.1)	1.0 (0.0-6.3)	<0.02
Area of vessels stained for TGF- β 1 μ m ² /mm Rbm	0 (0-545)	379 (0-2132)	324 (0-2882)	155 (0-4029)	<0.007
Percent of No. of Rbm vessels stained for TGF- β 1/mm Rbm x 100 [‡]	0 (0-26)	31 (0-121)	41 (0-311)	22 (0-114)	<0.03
Percent of area of Rbm vessels stained for TGF- β 1/mm Rbm x 100 [‡]	0 (0-24)	37 (0-194)	48 (0-503)	26 (0-324)	<0.02

*Kruskal-Wallis test, [†] All values are median (range)

[‡] Calculated as number and area of vessels respectively in the Rbm stained for TGF- β 1 divided by number and area of vessels stained for collagen IV x 100.

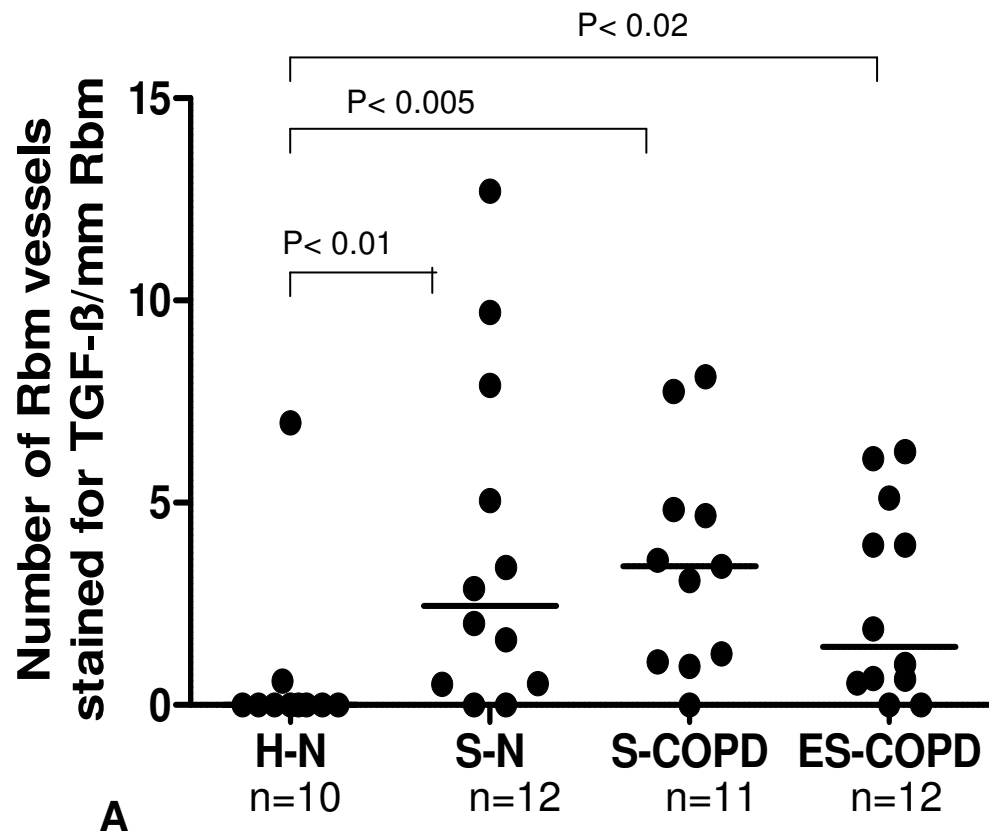


Figure 5-7A. *Vessels stained for TGF- β in the Rbm, number of vessels.*

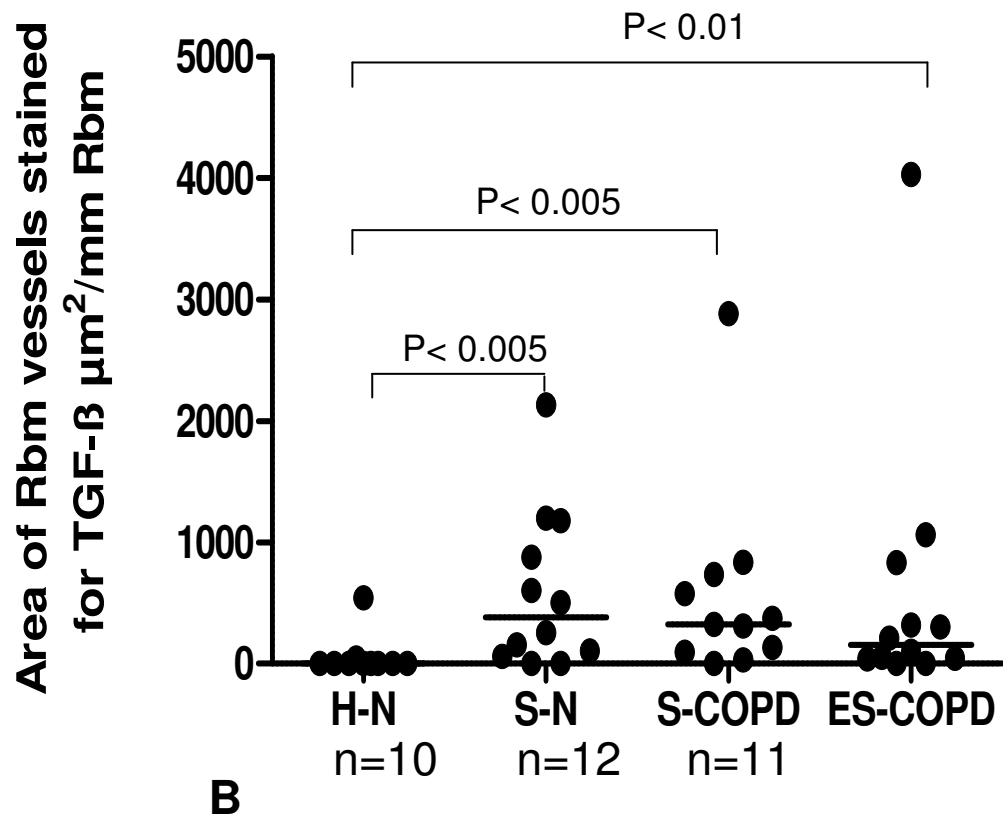


Figure 5-7B. Vessels stained for TGF- β in the Rbm, area of vessels.

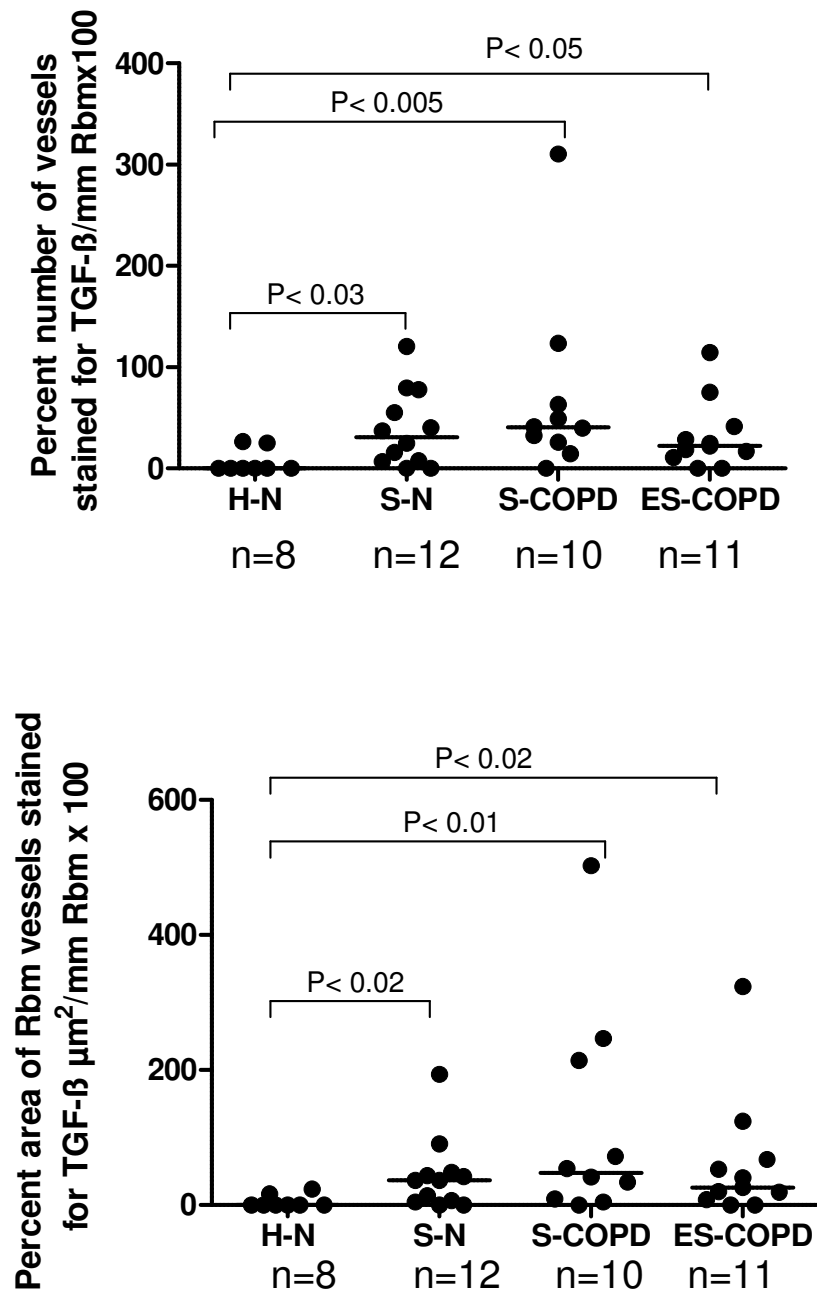
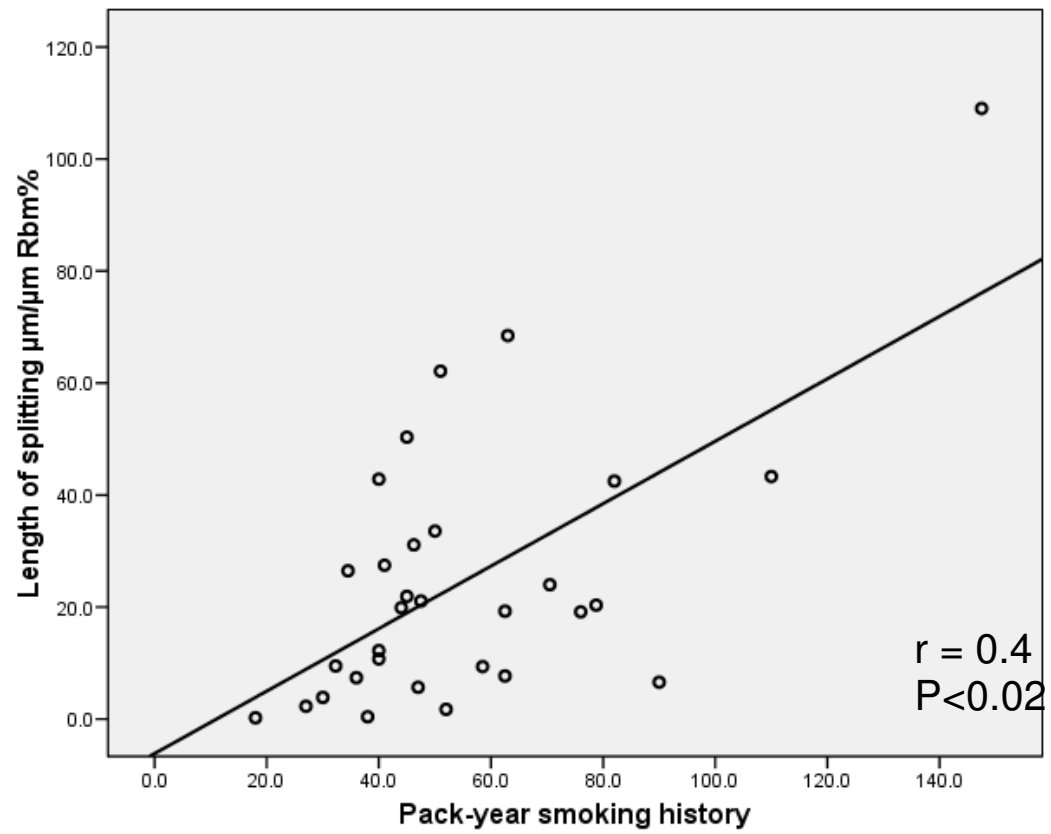
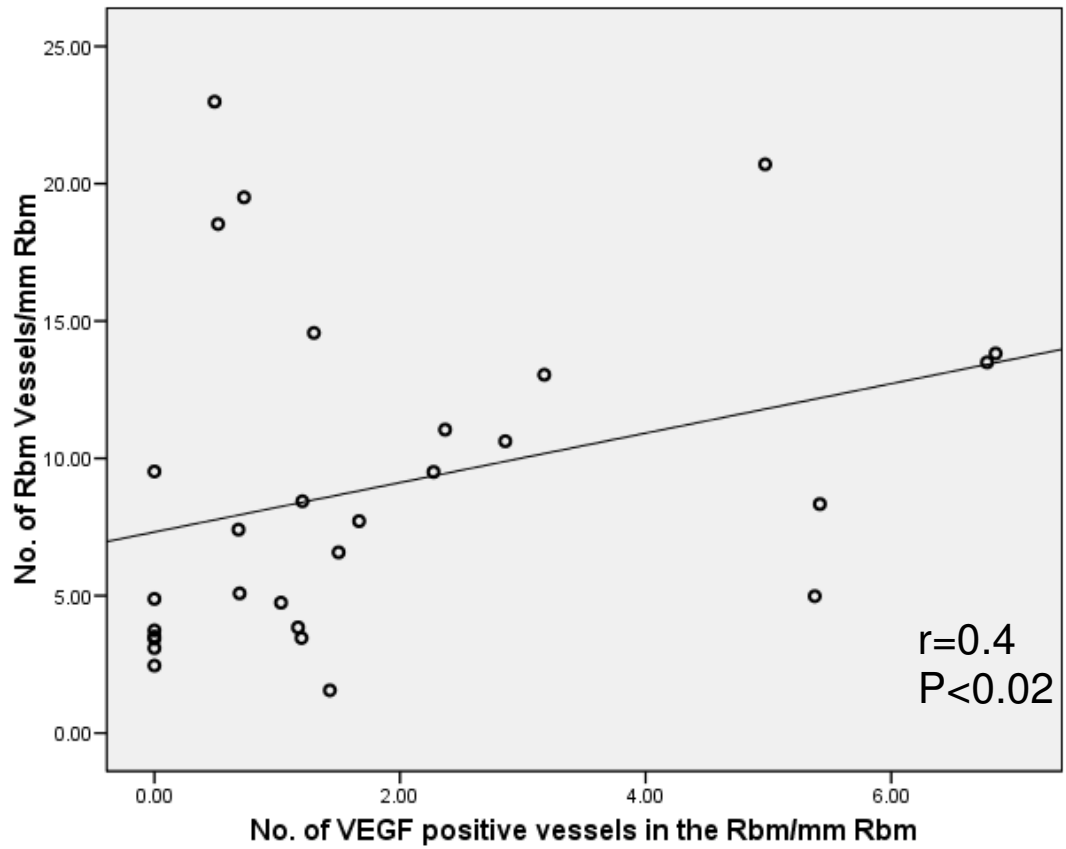


Figure 5-8: *Percent of vessels stained for TGF- β in the Rbm.*



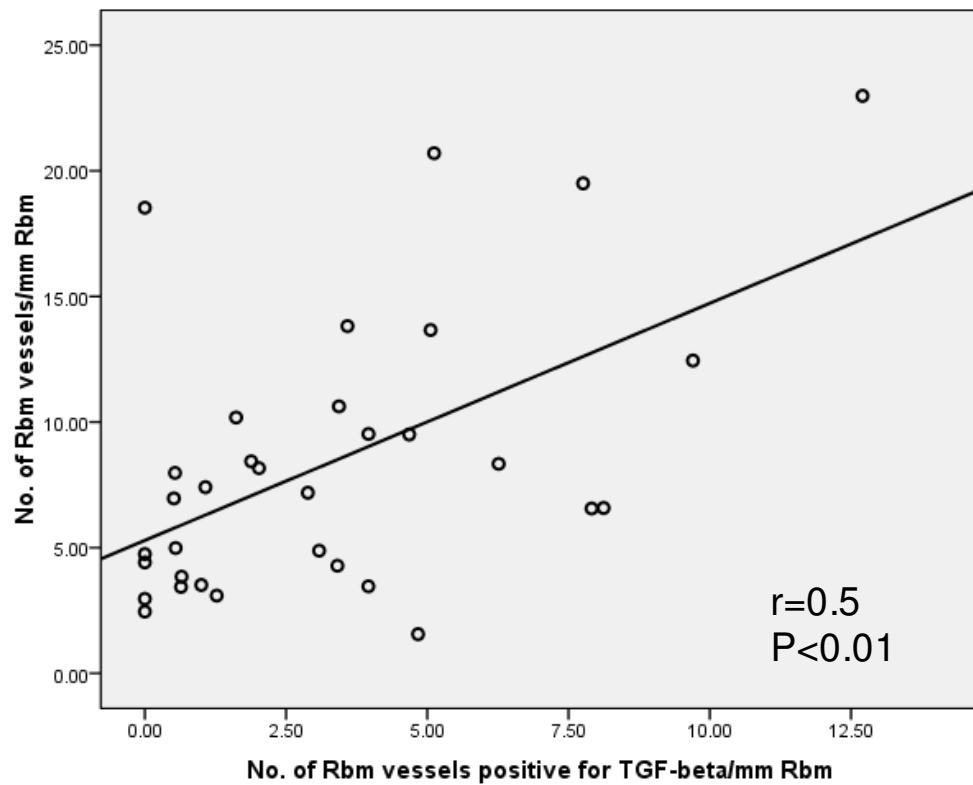
Both COPD groups, n=31

Figure 5-9. *Correlation between smoking history and length of Rbm splitting.*



Both COPD groups

Figure 5-10. *Correlation between Rbm vessels and vessels stained for VEGF (n=29).*



Both COPD and S-N groups together, n=33

Figure 5-11 A: Correlation between number of Rbm vessels stained for TGF- β and total number of vessels in the Rbm stained with Collagen IV antibody in both COPD groups and S-N group together.

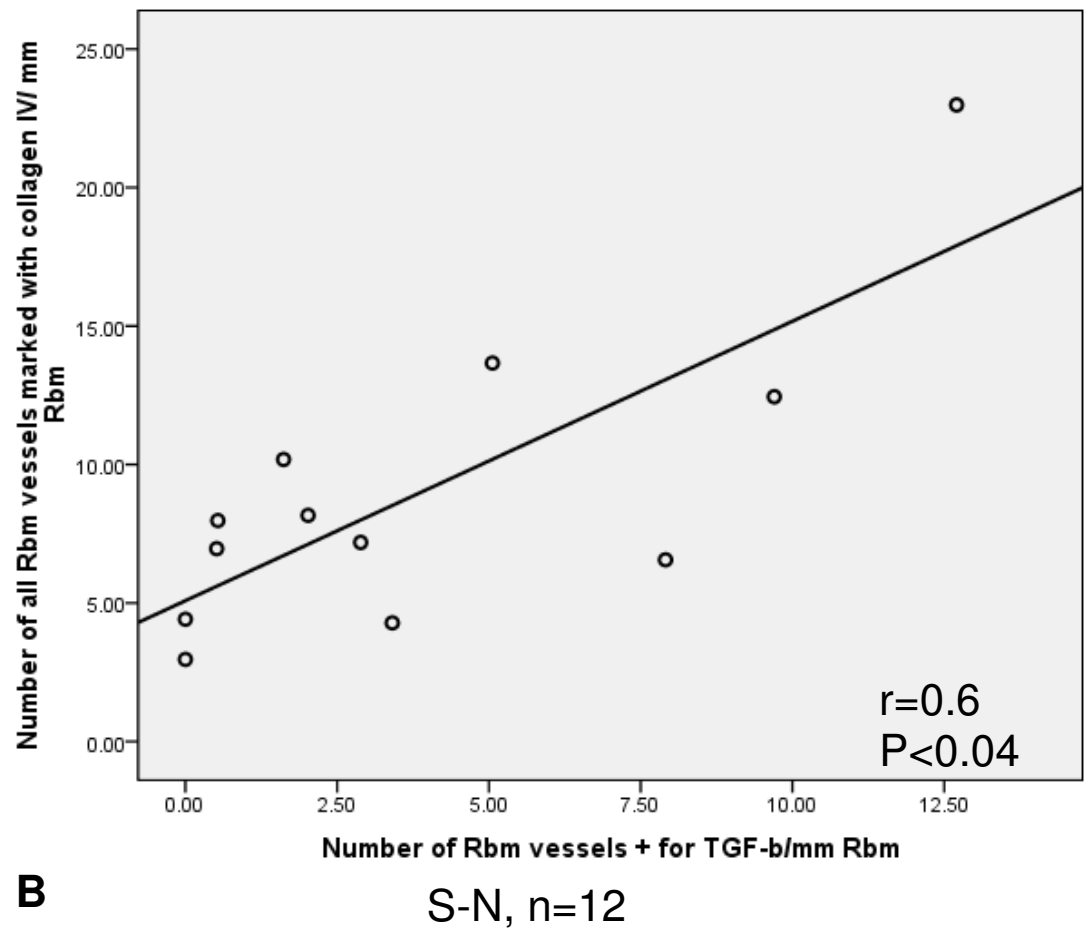


Figure 5-11 B. Correlation between number of Rbm vessels stained for TGF- β and total number of vessels in the Rbm in the S-N group.

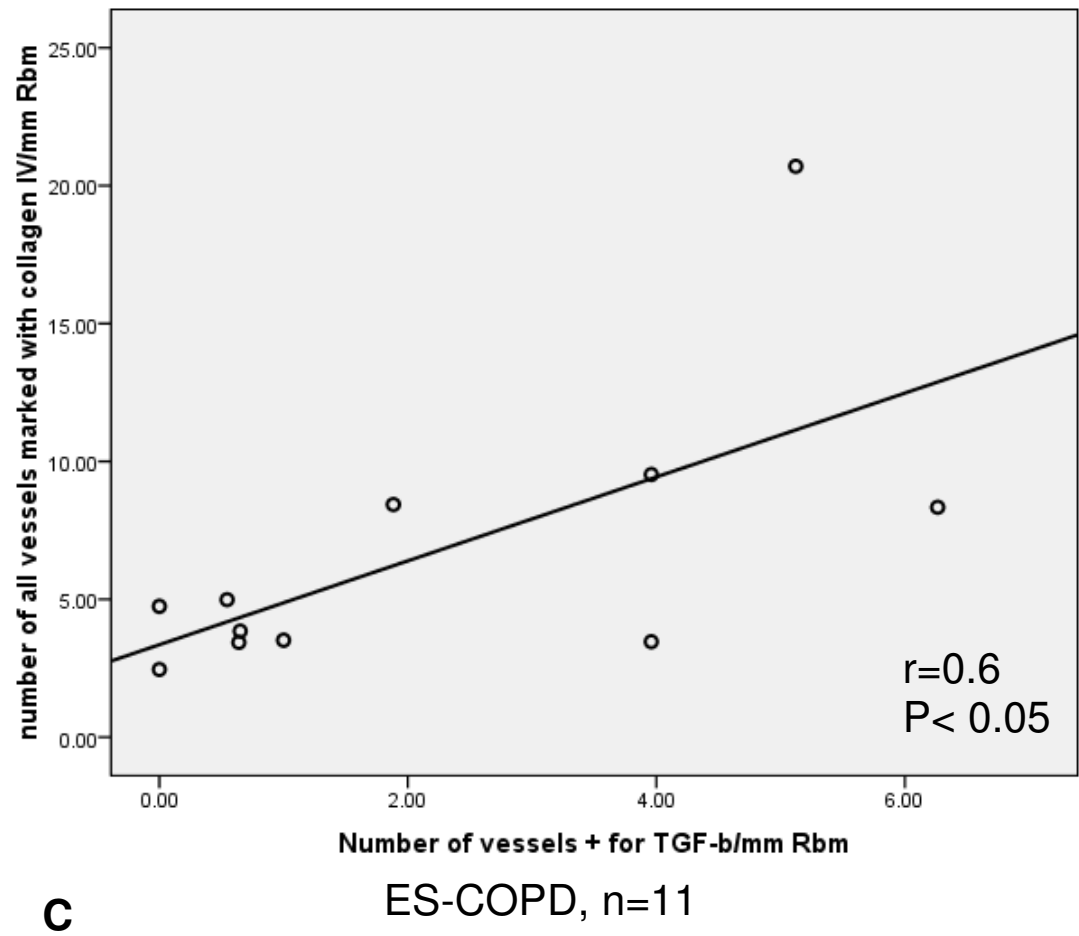
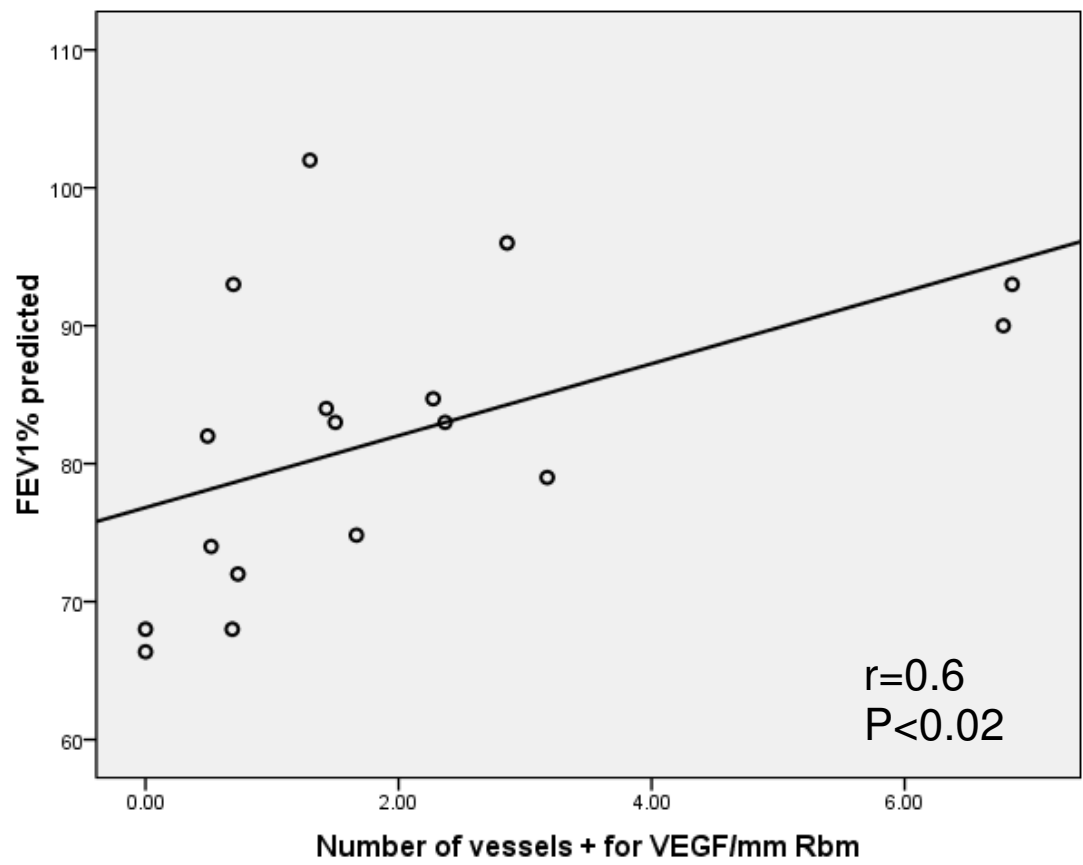


Figure 5-11 C. Correlation between number of Rbm vessels stained for TGF- β and total number of vessels in the Rbm in the ES-COPD group.



S-COPD, n=17

Figure 5-12. *Number of vessels stained for VEGF in S-COPD is related to FEV1% predicted.*

Correlations: The main correlations we found in my study can be summarised as below:

- We did not find any suggestion of a relationship between age and any of the pathological findings within groups; except for Rbm vascular area in the H-N group only (Tables 5-2 and 5-3).
- The pack-year history of smoking and degree of splitting of the Rbm were positively correlated ($r = 0.4$, $p < 0.02$) for the two COPD groups (Figure 5-9), but not in physiologically normal smokers.
- The total number of vessels in the Rbm correlated with number of vessels stained for VEGF in both COPD groups ($r = 0.4$, $p < 0.02$) (Figure 5-10).
- The number of Rbm vessels stained for TGF- β was related to total number of Rbm vessels in both COPD groups and S-N group together ($r = 0.5$, $p < 0.01$), S-N group ($r = 0.6$, $p < 0.04$) and ES-COPD ($r = 0.6$, $p < 0.05$) (Figure 5-11).
- The S-COPD group only showed positive correlations between FEV1% predicted and vessels positive for VEGF in the Rbm ($r = 0.6$, $p < 0.02$) (Figure 5-12) and also percentage number of vessels positive for VEGF ($r = 0.6$, $p < 0.01$).
- The S-COPD group also showed a positive correlation between vessel number in the LP and FVC% predicted ($r = 0.5$, $p < 0.05$).

Discussion:

This study has revealed novel aspects of airway remodelling in the large airways in smokers with or without COPD. We have attempted to differentiate effects of smoking from the changes specifically related to established COPD as defined by the GOLD initiative.

Our main results may be summarised as follows:

1. Rbm thickness, although quite variable in COPD, was not different between groups.

2. The Rbm was fragmented and had markedly increased splitting in smokers and COPD (Figure 1-3, Chapter 1), but changes were especially marked in current smoking COPD.
3. The Rbm was associated with hypervascularity in smokers with and without COPD but not in ES-COPD.
4. The LP was hypovascular in smokers but not in ES-COPD.
5. Vessel staining for VEGF and for TGF- β was increased in smokers and COPD, but again especially in current smokers with COPD.

We did not find a significant difference between groups in Rbm thickness. Previous studies have been contradictory. One group found thicker Rbm in COPD compared with controls, (Liesker et al., 2009) with both COPD and control groups in this study being ex-smokers except for 3 COPD subjects who were never smokers. Others have not found this difference (Chanez et al., 1997; Jeffery, 2004). Chanez et al.'s study showed that a subgroup of COPD with eosinophilic inflammation of airways had thicker Rbm than others (Chanez et al., 1997), suggesting that they were perhaps looking at an asthma subphenotype. Our studies by our group in COPD subjects with BDR did not show excessive eosinophils in the airways (D. W. Reid et al., 2008) or overt Rbm thickening. I found that Rbm thickness was not easy to measure accurately in COPD because of its fragmented appearance.

Indeed, main change in the Rbm in smokers and both COPD groups was marked fragmentation and in smokers only, hypervascularization. Both findings are novel and not previously published in the COPD literature. We propose that Rbm splitting could be the result of either new layers being formed by the epithelium or more likely, in the absence of Rbm thickening, degradation of the Rbm by proteolytic enzymes. Rbm splitting has been reported previously in the glomerular basement membrane and endothelial basement membrane of tubules in kidney transplant rejection. (Cornell,

Smith, & Colvin, 2008) (Ivanyi, Kemeny, Szederkenyi, Marofka, & Szenohradszky, 2001) Cornell *et al.* proposed that splitting is the consequence of repeated episodes of injury to the endothelium with new basement membrane layers formed by damaged endothelial cells as part of a repair process.

Smoking induces repeated injury to the airway epithelium. As Cornell *et al.* proposed for kidney rejection, this may cause epithelial repair with formation of new layers in the Rbm. This is compatible with the correlation of smoking history and length of splitting in our study and also explains the nonhomogeneity of the texture and thickness of the Rbm in smokers.

However, the presence of splitting may well represent a change or degradation in Rbm matrix proteins. Recently, differences have been described in the components of collagen and other proteins in the Rbm in a study comparing asthma, COPD and controls. (Liesker *et al.*, 2009) Change in proteinase activity, which has been shown in COPD, (Demedts *et al.*, 2005) may potentially explain this phenomenon. Our group has shown that the expression of MMP-9 in the Rbm is upregulated in smokers and in COPD. We showed that the number of cells stained for MMP-9 increased in the Rbm of smokers and COPD cases compared to controls (Sohal *et al.*, 2009), and proposed that these changes are a consequence of activation of Epithelial Mesenchymal Transition (EMT). Indeed, a fragmented Rbm is thought of in the EMT literature as a “hallmark” of the condition (Kalluri & Weinberg, 2009; Zeisberg & Neilson, 2009).

As discussed previously in Chapter Two, oxidative stress, delivered by cigarette smoke and inflammatory cells, activates a complex self-perpetuating pro-inflammatory and pro-remodelling intracellular mechanisms such as RAGE (see Chapter Two) and its ligands, NF- κ B, TGF- β and Twist. As Figure 2-20 in Chapter Two illustrates, ROS activate RAGE and NF- κ B. RAGE is upregulated at sites of inflammation and in a vicious cycle upregulates inflammatory cells. Besides, NF- κ B increases transcription of inflammatory genes and therefore intensifies inflammation in tissues, and inflammation in turn increases ROS production (P. J. Barnes, 2006). NF- κ B, an inducer of the “master-switch

transcription factor” Twist, is also related to remodelling changes through Twist (Pozharskaya et al., 2009). These mechanisms may continue in susceptible people, even after the insulting cause (smoking) disappears, and need specific investigation in COPD.

Current smokers, irrespective of their pulmonary function, had increased vessel numbers in and close to the Rbm. This pathological change may be reversible with smoking cessation, as the ES-COPD group was not different from H-N but was different from S-N and both current smokers together. This suggestive conclusion needs to be confirmed in a prospective smoking cessation study, which is now underway. We stained a number of matched slides with Factor VIII, which stains endothelium of blood vessels (Vrugt et al., 2000). Factor VIII staining confirmed that the structures stained by Collagen IV were indeed vessels (Figure 5-13).

We found more Rbm-associated vessels stained for VEGF in current smokers and COPD, but they were most marked in current smoking COPD subjects. VEGF- stained vessels correlated with the total number of Rbm-associated vessels in COPD only. VEGF is present in actively proliferating endothelium and is thought to be a marker of active angiogenesis (Ferrara et al.). Therefore, we suggest that angiogenesis is hyperactive in and close to the Rbm in current smokers and it is most active in current smokers with COPD. Angiogenesis does not seem to fully normalise with quitting. Our prospective, longitudinal smoking-cessation study will follow this up.

TGF- β staining showed increased vessel-related upregulation in the Rbm, again supporting active angiogenesis in this area. TGF- β has angiogenic activities (Jackson et al., 1997; Klagsbrun & D'Amore, 1991; Puxeddu et al., 2005). An in vivo study showed that it can induce angiogenesis (Roberts et al., 1986). All isoforms of TGF- β increased VEGF release by cultured human smooth muscle cells (F.-Q. Wen et al., 2003).

In contrast, there were fewer blood vessels in the LP in current smokers, but this was not found in ES-COPD. Hypovascularity of bronchial artery has been reported in old studies that used dye to examine the arterial system in the airways of COPD subjects {Paredi,

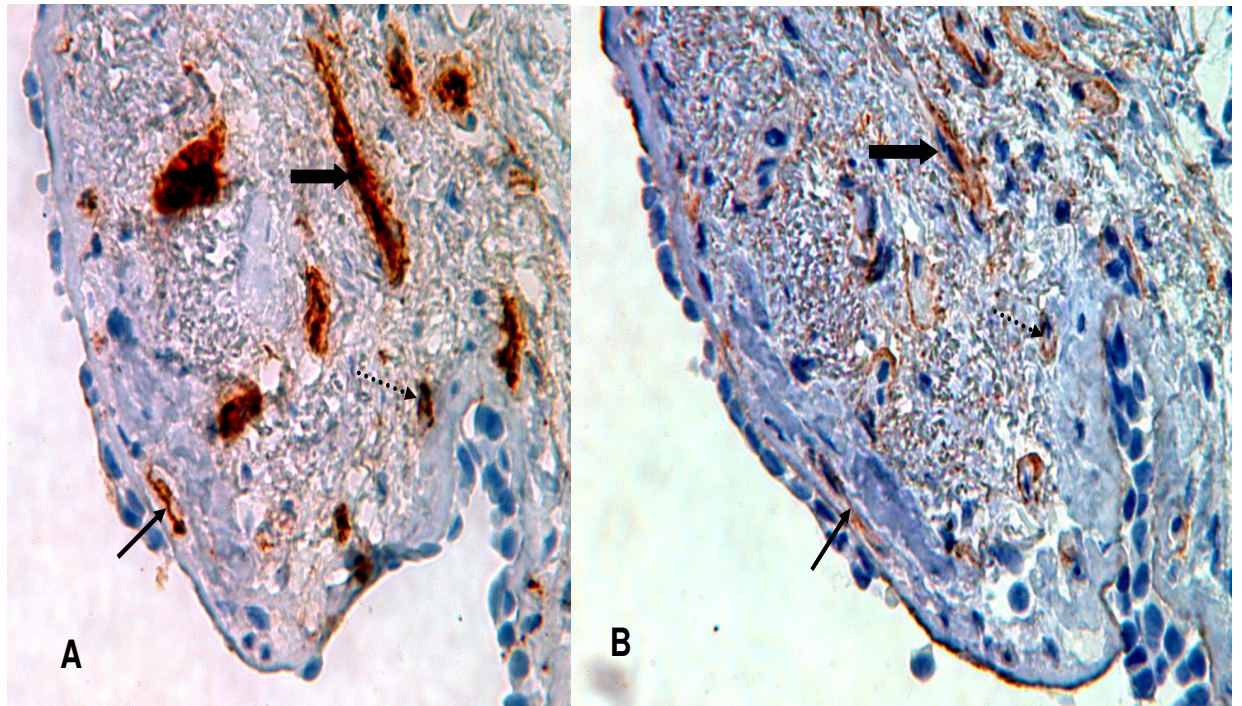


Figure 5-13. *Vessels stained with Factor VIII and collagen IV antibodies in same sample. A.* Vessels are marked with Factor VIII. **B.** Vessels are marked with collagen IV. Similar shaped arrows in both figures show same vessels stained with both antibodies. Current smoker COPD, X400.

Table 5-8. Summary of published studies about airway vascular remodelling in COPD and smokers

Study group	methods	Main results
(Zanini et al., 2009)	BB from: -10 exsmokers with moderate to severe COPD (group 1) -10 exsmokers with moderate to severe COPD on ICS (group 2) -8 control subjects (CS) with pulmonary nodules or haemoptysis -Collagen IV antibody	1. Number of vessels in the LP was not different between groups. 2. Vascular area in the LP was higher in group 2 compared to CS.
{ Calabrese, 2006 #94 }	BB from: -9 smokers with chronic bronchitis and moderate COPD and no emphysema (group 1) -9 smokers with normal lung function and chronic bronchitis (group 2) -8 control subjects (CS) -Collagen IV antibody	1. Number and area of vessels in the LP higher in groups 1&2 compared to CS. 2. Lower number of vessels in the LP in group 1 compared to group 2.
(Hashimoto et al., 2005)	Thoracotomy and resection of the lung from: -11 moderate COPD with “smoking history” -9 asthmatics -8 control subjects (CS) -All subjects had peripheral lung cancer -CD 31 antibody -Smooth muscle area was included in vessel counting	1. Higher vascular area in small airways in COPD compared to CS, but no difference in number of vessels. 2. No difference between COPD and CS in vascularity in the medium airways.
(Kuwano et al., 1993)	Thoracotomy and resection of the lung from: -15 post-mortem examinations in asthmatics (group 1) -15 mild COPD (group 2) -15 control subjects (CS) -groups 2&CS had peripheral lung cancer -peripheral airways were studied -trichrome staining	1. No difference in the number of vessels in the LP between groups.

Table 5-9. Summary of published studies about VEGF in COPD and smokers

Study group	Methods	Main results
(Zanini et al., 2009)	BB from: -10 exsmokers with moderate to severe COPD (group 1) -10 exsmokers with moderate to severe COPD on ICS (group 2) -8 control subjects (CS) with pulmonary nodules or haemoptysis	1.Number of VEGF positive cells in the LP was higher in group 1 compared to CS.
(Calabrese et al., 2006)	BB from: -9 smokers with chronic bronchitis and moderate COPD and no emphysema (group 1) -9 smokers with normal lung function and chronic bronchitis (group 2) -8 control subjects (CS)	1.Number of VEGF positive cells in the LP was higher in groups 1&2 compared to CS.
(Hashimoto et al., 2005)	Thoracotomy and resection of lung from: -11 moderate COPD with “history of smoking” -9 asthmatics -8 control subjects (CS) -All subjects had peripheral lung cancer -Smooth muscle area was included in vessel counting	1.No difference in VEGF positive cells between COPD and CS.
(Kranenburg et al., 2005)	Lung resection from: -14 current-/ex-smokers with mild to moderate COPD -14 current-/ex-smokers without COPD	1. Bronchial VEGF was higher in airway smooth muscle in COPD. 2. VEGF was higher in vascular smooth muscle cells in large airway in COPD. 3. VEGF was higher in bronchiolar epithelium and smooth muscle cells in COPD. 4. Higher VEGF in macrophages in small airways in COPD.
(H. Kanazawa & Yoshikawa, 2005)	Sputum from: -14 mild COPD -15 moderate COPD -16 severe COPD -12 very severe COPD -12 non-smoking control subjects	1. VEGF reduced with increasing severity of COPD.

Continues next page

Table 5-9. Summary of published studies about VEGF in COPD and smokers
(continued)

(K. Nagai et al., 2005)	BAL from -18 young smokers with various smoking history -23 old smokers with various smoking history -PCR for VEGF expression in alveolar macrophages and ELISA for VEGF level in BALF	1.mRNA VEGF reduced in current smokers compared to nonsmokers in old subjects. 2.BALF VEGF reduced in current smokers compared to non smokers in all age groups
(Kanazawa et al., 2003)	Sputum from: -25 COPD with emphysema -19 COPD with chronic bronchitis -15 mixed type COPD -20 nonsmoking asthma -11 nonsmoking control subjects (CS)	1. Higher VEGF in bronchitic COPD compared to CS. 2. Less VEGF in COPD with emphysema compared to CS. 3. No difference between mixed type and CS.
(Koyama et al., 2002)	BALF from: -11 current smokers -16 nonsmokers -35 pulmonary fibrosis -14 sarcoidosis	1. Reduced VEGF in smokers compared to nonsmoking volunteers and smokers with lung disease compared to nonsmokers with lung disease.
(Kasahara et al., 2001)	Lung resection from: -6 current smoker patients with severe emphysema -11 non-/ex-smokers (CS)	1. VEGF was reduced in lung tissue in emphysema.

2009 #429}(Cudkowicz & Armstrong, 1953) (See Chapter Seven, General Discussion for more details). There have been few previous studies investigating vascular changes in BB taken from COPD patients, and none to my knowledge that have differentiated the Rbm and the LP. For a summary of published studies about vascular remodelling and VEGF level in smokers and COPD please see Tables 5-8 and 5-9. Below, I will now summarise some of the most relevant conclusions from these papers.

Calabrese *et al.* in a study on bronchoscopically-obtained biopsies reported more vessels in the LP of smokers, and concluded that angiogenesis is a part of airway remodelling in smokers. They did not find any relationship between remodelling changes and lung function or clinical manifestations (Calabrese et al., 2006). Another recent study from Italy found a larger vascular area in BB from ex-smokers with moderate to severe COPD compared to control subjects, but the number of vessels was not different between groups (Zanini et al., 2009); Zanini et al. did not have a physiologically-normal smoking control group.

A potential explanation for the findings from these papers, which appear to contrast with our own, would be the different selection criteria employed. For example, Calabrese et al. recruited smokers with normal lung function or COPD, but all had clinical criteria of “chronic bronchitis” and they excluded subjects with emphysema. Chronic bronchitis was almost completely absent in our S-N subjects; and we did not exclude subjects with emphysema in our COPD groups (Table 5-1). In general, chronic productive cough is a lesser feature of COPD in Australia, and we did not select on this basis. Indeed, we attempted to recruit a “typical” mild-moderate COPD group. Zanini *et al.* recruited moderate to severe COPD subjects who had quit for more than 10 years and they did not study current smoker COPD subjects. In our study current smokers showed most changes compared to the control group. The Zanini et al study may be confounded by 10 years being long enough for smoking-related changes to largely resolve.

We separately counted vessels in the Rbm and LP. However, if the Rbm- associated vessels were added to vessels in the LP (i.e. total mucosal vessels) we still found fewer

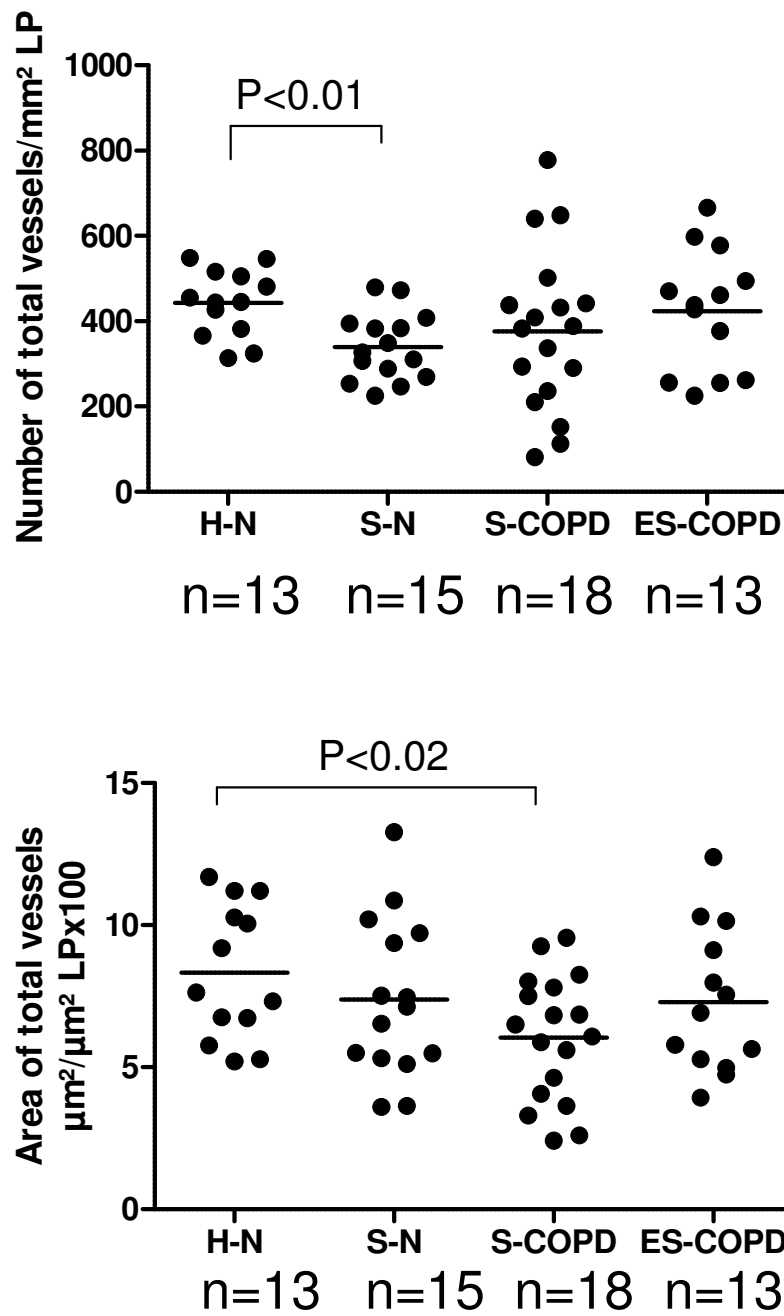


Figure 5-14. Total mucosal vessels (Rbm and LP vessels together) compared in study groups.

vessels overall in the mucosa in current smokers. There were fewer vessels in S-N vs. H-N [mean (SD) 340(79) vs. 443 (78), $p<0.01$] and less vascular area in S-COPD vs. H-N [mean (SD) 6.0 (2.2) vs. 8.3 (2.4), $p<0.02$] (Figure 5-14).

There are other studies that have examined airway vascularity in COPD but these used subjects with peripheral lung cancer to study mainly small airways in lung resection specimens. (Hashimoto et al., 2005; Kuwano et al., 1993) Hashimoto et al. found larger vascular area in the compartment between sub-epithelial BM and the outer border of smooth muscle in small airways in COPD subjects when compared with nonsmokers without airway disease. This difference was not seen in larger airways with an internal diameter of 2-5 mm. In contrast, Kuwano *et al.* did not find a significant difference in vessel density in the mucosa of peripheral airways in subjects with mild COPD when compared to the mucosa of controls without airway disease.

The reason for our finding of hypovascularity of the LP in smokers can not be directly explained by the design of our study. However, respiratory tract VEGF reduction in smokers has been previously reported (K. Nagai et al., 2005) (Koyama et al., 2002). Hypovascularity of the LP in current smokers may be analogous to the observation that down-regulated VEGF is present within the lung parenchyma and may be the primary cause of the development of emphysema (Wagner, 2003) (Kasahara et al., 2001).

Our current study did not find reduced VEGF activity or in the LP in the current smoker groups, with percentage of vessels in the LP stained for VEGF not significantly different between the groups. However, an explanation for this apparent paradox may be that VEGF is functionally unavailable for new vessel formation in the presence of cigarette smoke and there are data to support this possibility. Thus, a study on human umbilical vein endothelial cells showed that cigarette smoke extract blocked endothelial cell migration in response to VEGF and inhibited VEGF-induced tube formation. This blocking mechanism was associated with increased production of ROS (Michaud, Dussault, Groleau, Haddad, & Rivard, 2006). The finding of normal vessels in ES-COPD without a change in vessel-associated VEGF in the LP in our study supports this

idea. More studies of the angiopoietic systems in smokers are indicated. Deprivation of other angiogenic factors, such as angiopoietin-1 and/or down-regulation of endothelial VEGF receptors also needs to be considered and studied (Bryce N. Feltis et al., 2006).

Whatever the mechanism, hypovascularity of the LP is a smoking effect that may be reversible with quitting, but a specific longitudinal study is needed to confirm that. The potential physiological consequences or associated changes on immune cell and water flux in the LP, and any related changes in physical/structural characteristics also need investigation.

The strong relationship between Rbm vessel-related VEGF and better FEV1% predicted in the S-COPD group that was found is interesting. There is some evidence that remodelling may have a protective effect. (Bergeron & Boulet, 2006; A. Nagai, West, & Thurlbeck, 1985), (Lambert, Codd, Alley, & Pack, 1994) Angiogenesis in the Rbm, perhaps by increasing airway stiffness and resisting dynamic compression, may provide protective effects to COPD. This could be confirmed by assessment of airway distensibility (Johns, Wilson, Harding, & Walters, 2000). On the other hand, the relationship between lower number of LP vessels and lower FVC, may suggest increased air trapping due to more dynamic compression of small airways, again compatible with a mechanical consequence of the LP hypovascularity at least in that compartment.

Limitations of the cross-sectional study are summarised as follows:

1. COPD subjects were significantly older compared to non-COPD subjects. However, the age range in COPD was wide and age did not seem to influence the main findings (Tables 5-2 and 5-3).
2. There was a difference in pack-year smoking history between smokers with normal lung function and COPD subjects.

As far as I can determine, nobody has studied the effects of ageing on airway remodelling in BB from COPD. However, we may presume that the longer the smoking

history, the more pathological changes there will be. Therefore, older people with longer history of smoking and higher pack-year smoking history may have more advanced pathological changes. This would confound the differentiation between disease effect and smoking in our COPD subjects.

The effects of ageing and smoking have actually been studied on VEGF. Studies showed that both have effects on reducing VEGF level in BALF (Koyama et al., 2002; K. Nagai et al., 2005). But, to my knowledge, there is no previous report about the effects of these two factors on VEGF staining or activity in BB.

Theoretically, to avoid these limitations we needed to find age-matched subjects with the same smoking history as our COPD subjects. Or we needed to carry out a much larger study with more subjects and a wide range of age and pack-year smoking history categories to examine the effect of these two factors on airway tissue. However, as these subjects would require bronchoscopy, which is an invasive procedure, neither strategy is really feasible. The analysis we have done shows no suggestion at all that the pathological changes of Rbm splitting or the vascular changes in either Rbm or LP compartments were age-related and this is highly reassuring, short of doing more definitive studies.

Conclusions:

This study examined novel aspects of Rbm and vascular remodelling in the large airway biopsies in current smokers with normal lung function and patients with established mild to moderate COPD. Most changes seemed related predominantly to smoking, but some were most marked in current smoking COPD patients, suggesting additive effects in this situation. Vessel changes seemed reversible with quitting smoking whereas Rbm fragmentation did not. This needs follow up confirmation in longitudinal quitting studies. Vascular changes in the Rbm and LP were in opposite directions in current smoking groups, i.e. the Rbm was hypervascular and the LP was hypovascular. Hypervascularity of the Rbm was associated with increased VEGF expression that was in turn positively related to better lung function in current smokers with COPD. TGF- β

activity was also intensified. These findings are likely to indicate active angiogenesis. Further investigations are needed to study the VEGF system and receptors in greater depth, and other angiogenic factors that may contribute to vascular redistribution in the airways of smokers with or without COPD. As well as longitudinal studies to assess the effects of smoking cessation, disease-modifying therapy such as inhaled corticosteroids need to be assessed on airway remodelling in COPD. These studies will help clarify the clinical and pathophysiological significance of our findings.

Chapter Six

Effects of Inhaled Corticosteroid Therapy on Airway Remodelling in COPD: A Longitudinal Study

Abstract:

Introduction: Chronic inflammation of airways and airway remodelling are components of COPD. In chapter 5, I reported reticular basement membrane (Rbm) and vascular remodelling in bronchial biopsies from smokers with or without COPD. Our knowledge about the effects of inhaled corticosteroids on large airway remodelling in this disease is very limited.

Objective: To assess the effects of inhaled corticosteroids on the novel airway remodelling characteristics in bronchial biopsies from COPD.

Methods: 34 subjects with mild to moderate COPD were allocated 2:1 randomly to active and placebo treatment in a longitudinal and double blind clinical trial comparing 6 months of fluticasone propionate (FP, 500 µg twice daily) with placebo. Lung function studies and bronchoscopic biopsies were performed before and after intervention and

samples were compared to assess the effects of treatment on reticular basement membrane (Rbm) and vascular remodelling.

Results: The Rbm showed improvement in splitting in the active treatment group [median (range) 19.1 (0.2-42.8) vs. 2.6 (0.0-88.6) before and after treatment respectively $p<0.03$] but did not change with placebo [median (range) 24.0 (6.6-109.0) vs. 26.9 (2.5-48.5), p not significant]. The length of Rbm splitting was significantly shorter in the FP group compared to the placebo group at the end of the treatment [median (range) 2.6 (0.0-88.6) for FP vs. 26.9 (2.5-48.5) for placebo, $p<0.05$]. Vessels in the Rbm and LP did not change with treatment in either group. There was improvement in lung function in the active treatment group and deterioration in the placebo group, but lung function and pathological changes did not correlate.

Conclusion: Six months inhaled corticosteroid treatment improves lung function and is effective in Rbm remodelling in mild to moderate COPD but does not change mucosal vessel remodelling.

Introduction:

ICS are commonly used in the treatment of both asthma and COPD. ICS are the first-line therapy in long-term management of asthma (P.J Barnes, 1998; Orsida et al., 1999). They control symptoms, decrease the frequency and severity of exacerbations and improve lung function in asthma (P.J Barnes, 1998). The effects of ICS in airway remodelling in asthma have also been evaluated. Rbm thickening, hypervascularity of the LP and increased angiogenic activity all have been reported to respond to treatment with ICS (Olivieri et al., 1997; C. Ward et al., 2002) (Orsida et al., 1999) (B. N. Feltis et al., 2007).

The clinical response to ICS in COPD is not as well established as in asthma (E. H. Walters, Reid, Johns, & Ward, 2007) (GOLD, 2007) (B. R. Celli et al., 2004), and some authors are distinctly against their use in COPD (Rodrigo et al., 2009) (Suisse & Barnes, 2009). However, ICS have become a part of standard treatment in severe COPD (GOLD, 2007) (B. R. Celli et al., 2004) on the basis of results from large multi-centre studies. These studies have shown short-term improvement in lung function and longer-term benefits in terms of quality of life (QOL) and mortality (see chapter two) (Burge et al., 2000; P. Calverley et al., 2003; Ferguson et al., 2006; Mahler et al., 2002; Paggiaro et al., 1998).

There are some reports about the effects of ICS on inflammatory cells in COPD (see Chapter Two for more details). Reid et al. has shown recently that ICS compared to placebo reduced macrophage numbers in BB and neutrophil numbers in BAL from COPD subjects. In the FP group there were decreases in mast cells and CD8+ cells in BB (D. W. Reid et al., 2008). Another placebo controlled trial showed reduction of CD8:CD4 ratio with FP in BB from COPD subjects (Hattotuwa et al., 2002). A larger study compared salmeterol (a long-acting beta agonist) plus FP combination (SFC) to placebo, and found that the number of CD8+ cells in BB from COPD subjects reduced with the combined treatment (N. C. Barnes et al., 2006). Another study compared SFC to FP alone and to placebo in the BBs from COPD subjects. This study found a

significant reduction of CD8+ T-cells and CD68+ macrophages in the combined therapy group, but not with FP alone, compared to placebo (Bourbeau et al., 2007).

Information available in the literature about the effects of ICS on structural changes of airway remodelling in COPD is extremely limited. Zanini et al. carried out a *cross-sectional study* to compare BB from ex-smokers with COPD that either were or were not on ICS to a control group. They reported that in COPD group that was not on ICS the vascular area, VEGF positive cells and TGF- β positive cells in the LP were greater than the other groups. They suggested that vascular remodelling in COPD may respond to ICS (Zanini et al., 2009). Obviously longitudinal studies are required to properly evaluate the effects of ICS on airway remodelling in COPD.

In Chapters One and Five, I explained that our cross-sectional study found hypervascularity of the Rbm and hypovascularity of the LP in current smokers with or without COPD and also fragmentation and splitting of the Rbm in COPD subjects and current smokers with normal lung function. The study also found that vessel staining for VEGF and TGF- β was increased in the Rbm in current smokers.

We therefore undertook a randomised controlled clinical trial to compare the effects of ICS with placebo on airway remodelling in BB from COPD.

Aim: To evaluate the effects of ICS on airway remodelling in BB from COPD subjects.

Methods:

Study: These data were obtained from the same study published by Reid et al (D. W. Reid et al., 2008). This was a double blind, randomised, placebo controlled clinical trial. Figure 6-1 explains the study design. After a 2 weeks run-in period, baseline assessments including spirometry and bronchoscopy with airway biopsy were performed. Then, using a computer generated random numbers table, the participants were randomised 2:1 to fluticasone propionate (FP) (Accuhaler; Glaxo-Wellcome, Middlesex, UK) 0.5 mg/twice daily or placebo groups. Identical multi-dose dry powder

inhaler devices were used to deliver both medications. After 6 months of treatment, lung function study and bronchoscopic biopsies were performed again.

Subjects: 34 Subjects with COPD were recruited through advertisement and as above, were allocated randomly 2:1 to active and placebo treatments. (For details of inclusion/exclusion criteria etc. please see Chapter Three, Materials and Methods).

This study was approved by the Human Research Ethics Committee (Tasmania) Network. All subjects provided written informed consent.

Details of lung function studies, *FOB and endobronchial biopsying, tissue processing, quantifications and statistical analysis* are also presented in Chapter Three. Essentially, Rbm splitting, Rbm vessels, vessels in the LP, vessels stained with VEGF and vessels stained with TGF- β were assessed before and after treatment. As already described, all slides were coded by our laboratory manager and I was blinded to the treatment groups throughout the measurements and analyses.

Results:

Thirty-four subjects (23 FP and 11 placebo) were recruited (Figure 6-2). Of those in the FP group, 3 subjects withdrew because of side effects, one subject had a severe acute exacerbation needing systemic steroids and one participant admitted lack of adherence. In the placebo group, one subject refused second bronchoscopy and two subjects did not have enough paired tissues for comparison. Of the remaining 26 (18 FP and 8 placebo) subjects, 22 (15 FP and 7 placebo) had enough paired tissues for Collagen IV staining and 23 (16 FP and 7 placebo) had enough paired tissues for both VEGF and TGF- β staining (Table 6-1). As said before in Chapter One, there was an intense background staining with TGF- β antibody; thus I could only assess 17 (13 FP and 4 placebo) paired tissues for TGF- β study.

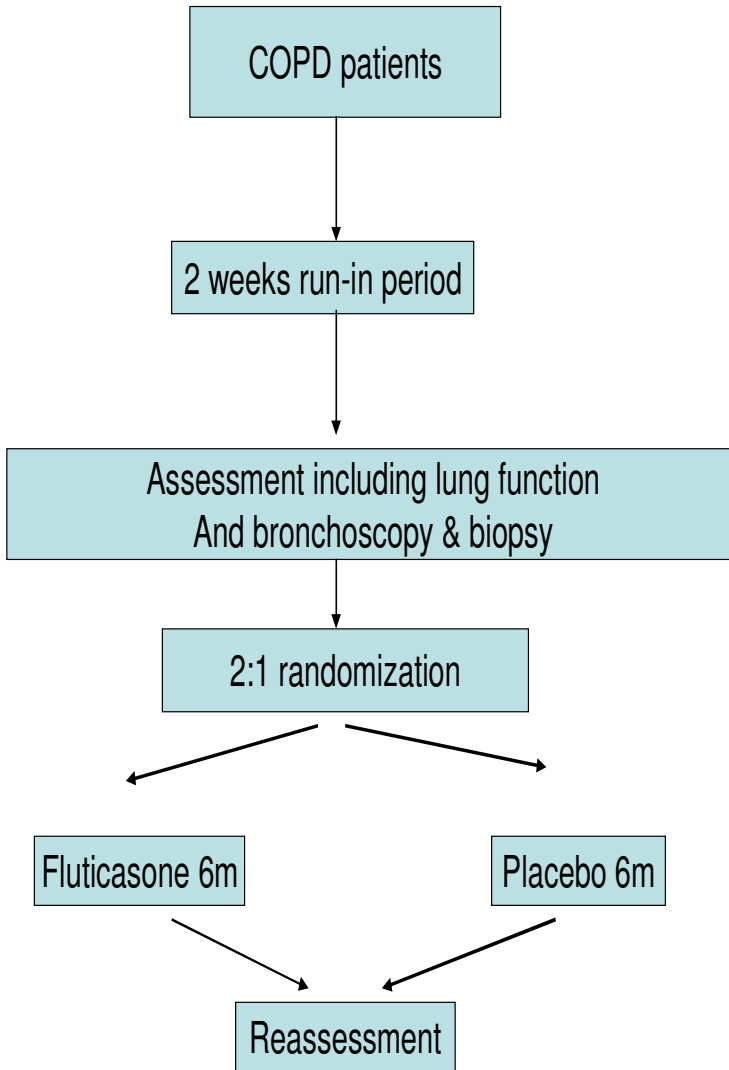


Figure 6-1. *Study design.*

Demographics of the study groups are presented in Table 6-2.

There were no significant differences between groups in demographics and lung function parameters (Table 6-2) and remodelling features and quantitated indices of interest before intervention (Table 6-3).

Table 6-4 summarises the results of the study.

The aggregate lengths of splitting of the Rbm reduced significantly in the FP group [median (range) 19.1 (0.2-42.8) before vs. 2.6 (0.0-88.6) after treatment, $p < 0.03$] but not in the placebo group [median (range) 24.0 (6.6-109) before vs. 26.9 (2.5-48.5) after, $p = 0.4$] (Table 6-4 and Figure 6-3). After treatment, the active treatment arm had significantly shorter splitting than the placebo group, [median (range) 2.6 (0.0-88.6) vs. 26.9 (2.5-48.5), $p < 0.05$]. The length of splits “normalised” after treatment with FP but did not change in the placebo group [median (range) 2.6 (0.0-88.6) after treatment with FP vs. 5.3 (0.0-21.4) H-N, $p = 0.7$] (Figure 6-4). The length of splits remained significantly greater in the placebo group after treatment compared with H-N [median (range) 26.9 (2.5-48.5) vs. 5.3 (0.0-21.4), $p < 0.02$] (Figure 6-4).

The vessels in the Rbm (Table 6-4 and Figure 6-5) did not change significantly with treatment in either group [median (range) for number of vessels in the Rbm/mm Rbm 8.7 (3.1-23.0) before vs. 6.4 (0.0-18.0) after FP, $p = 0.1$ and 8.3 (3.7-13.0) before vs. 1.9 (0.7-13.6) after placebo, $p = 0.3$; for area of vessels in the Rbm $\mu\text{m}^2/\text{mm Rbm}$ 895 (195-2159) before vs. 882 (0-2731) after FP, $p = 0.8$ and 574 (395-1224) before vs. 477 (104-1902) after placebo, $p = 0.2$] neither did *the vessels in the LP* [mean (SD) for number of vessels in the LP/ mm^2 LP 271 (146) before vs. 259 (125) after FP, $p = 0.7$ and 247 (81) before vs. 235 (78) after placebo, $p = 0.8$ and for area of vessels in the LP [$(\mu\text{m}^2/\mu\text{m}^2\text{LP}) \times 100$] 5.8 (2.1) before vs. 5.9 (3.2) after FP, $p = 0.9$ and 5.8 (3.4) before vs. 4.8 (1.9) after placebo, $p = 0.5$].

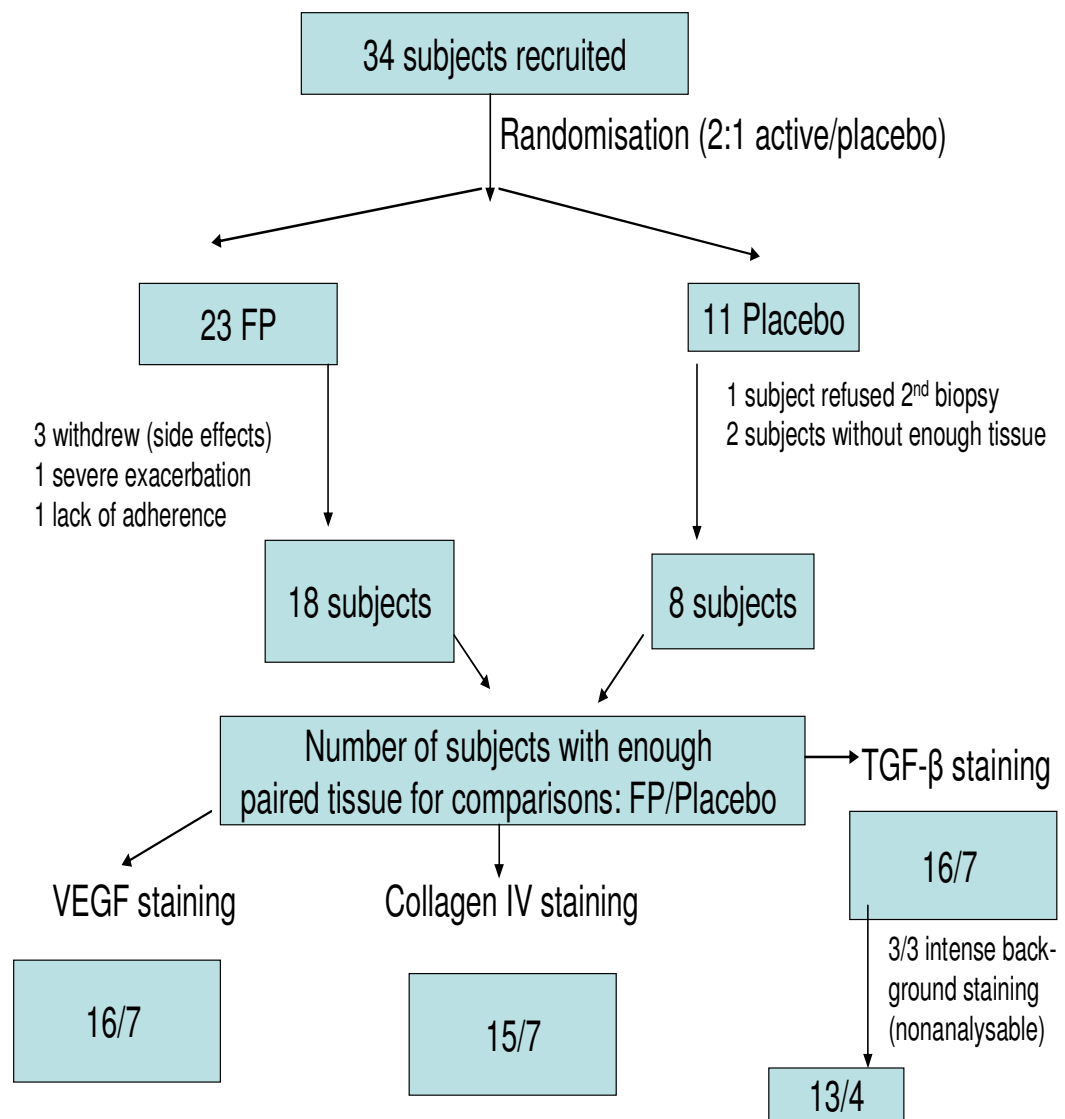


Figure 6-2. *Study subjects.*

Table 6-1. Data on the twenty-six subjects in the longitudinal study, their demographics, the treatment group for each subject and the analyses their tissues were used in: this is to emphasise that unfortunately for logistic reasons the numbers of subjects per analysis are not fully uniform*

Number	Gender	age	smoking	treatment	Studies†
1	Male	61	Ex	FP	C4,VE,TG
2	Male	58	Current	FP	C4,VE,TG
3	Female	53	Ex	FP	C4,VE,TG
4	Male	67	Ex	FP	C4,~~,~~
5	Male	69	Current	FP	C4,VE,~~
6	Male	67	Current	FP	C4,VE,TG
7	Female	60	Ex	FP	C4,VE,TG
8	Female	61	Current	FP	C4,VE,TG
9	Male	48	Current	FP	C4,~~,~~
10	Male	58	Ex	FP	C4,VE,~~
11	Male	63	Current	FP	C4,VE,TG
12	Male	67	Current	FP	C4,VE,~~
13	Male	53	Current	FP	C4,VE,TG
14	Male	55	Current	FP	C4,VE,TG
15	Female	57	Current	FP	C4,VE,TG
16	Female	60	Current	FP	~~,VE,TG
17	Male	57	Ex	FP	~~,VE,TG
18	Female	63	Ex	FP	~~,VE,TG
1	Male	69	Ex	Placebo	C4,VE,~~
2	Male	64	Ex	Placebo	C4,VE,TG
3	Male	69	Ex	Placebo	C4,VE,~~
4	Female	56	Ex	Placebo	C4,VE,~~
5	Female	64	Current	Placebo	C4,~~,~~
6	Male	66	Ex	Placebo	C4,VE,TG
7	Male	61	Current	Placebo	C4,VE,TG
8	Female	60	Ex	Placebo	~~,VE,TG

*Current = current smoker, EX = Ex-smoker, FP = Fluticasone propionate

† Analyses are coded as: C4 = Collagen IV, VE = VEGF, TG = TGF- β , ~~ = Not enough paired tissues available for the analysis; therefore (C4, VE, ~~) means there was not enough tissue available for a fully paired TGF- β analysis.

Table 6-2. Demographics

Groups (numbers)	FP (n=23)	Placebo (n=11)	P value [†]
Age years*	61 (46-69)	61 (52-69)	0.6 (NS) ¹
Female/Male	9/14	4/7	0.9 (NS) [‡]
Current smoker/Ex-smoker	13/10	4/7	0.3 (NS) [‡]
Pack-year smoking history*	44 (18-150)	51 (22-148)	0.4 (NS) [¶]
FEV1% predicted*	82 (55-112)	79 (54-94)	0.7 (NS) ¹
FVC% predicted*	109 (87-151)	116 (91-135)	0.9 (NS) ¹
FER*	59 (41-68)	57 (38-68)	0.7 (NS) ¹
DLCO% predicted*	65 (44-87)	66 (45-90)	0.9 (NS) ¹
IC, Liter*	3.0 (1.8-4.5)	3.0 (1.8-3.7)	1.0 (NS) ¹
BDR%*	16 (0-34)	10 (3-23)	0.6 (NS) ¹

* Median (range)

[†] NS = not significant[‡] Chi-square test, [¶] Mann-Whitney test, ¹ Independent-samples t-test

Table 6-3. Comparison of baseline Rbm and vessel measurements between the two groups

Groups	FP	Placebo	P value [‡]
Length of Rbm splitting/Rbm length x100, median (range) [¶]	19.1 (0.2-42.8)	24.0 (6.6-109.0)	0.2 (NS)*
No. of Rbm vessels/mm Rbm, median (range) [¶]	8.7 (3.1-23)	8.3 (3.7-13)	0.5 (NS)*
Area of Rbm vessels $\mu\text{m}^2/\text{mm}$ Rbm, median (range) [¶]	895 (198-2159)	574 (395-1224)	0.5 (NS) [†]
Density of LP vessels No./ mm^2 , mean(SD) [¶]	271 (146)	247 (81)	0.7 (NS) [†]
%Vascular area in LP $\mu\text{m}^2/\mu\text{m}^2 \times 100$, mean (SD) [¶]	5.8 (2.1)	5.8 (3.4)	1.0 (NS) [†]
No. of vessels in the Rbm stained for VEGF/mm Rbm, median (range) [•]	0.6 (0.0-5.4)	1.0 (0.0-5.4)	0.9 (NS)*
Area of vessels in the Rbm stained for VEGF $\mu\text{m}^2/\text{mm}$ Rbm, median (range) [•]	22 (0.0-746)	28 (0.0-534)	0.7 (NS)*
No. of Rbm vessels stained for TGF- β /mm Rbm, median (range) [°]	1.3 (0.0-8.1)	2.5 (0.7-4.0)	0.9 (NS)
Area of Rbm vessels stained for TGF- β /mm Rbm, median (range) [°]	135 (0-4029)	255 (42-311)	0.9 (NS)

* Mann-Whitney test, [†] t-test

[‡] NS = not significant

[¶] Number of subjects for FP vs. placebo: 15 vs. 7

[•] Number of subjects for FP vs. placebo: 16 vs. 7

[°] Number of subjects for FP vs. placebo: 13 vs. 4

Table 6-4. Summary of the effects of intervention for both groups*

	FP 1 st time point	FP 2 nd time point	P value ¹	Placebo 1 st time point	Placebo 2 nd time point	P value ¹
Length of Rbm splitting/Rbm length x100*	19.1 (0.2- 42.8)	2.6 (0.0- 88.6)	<0.03	24.0 (6.6- 109.0)	26.9 (2.5- 48.5)	0.4 (NS) [‡]
No. of Rbm vessels/mm Rbm*	8.7 (3.1- 23.0)	6.4 (0.0- 18.0)	0.1 (NS)	8.3 (3.7- 13.0)	1.9 (0.7- 13.6)	0.3 (NS) [‡]
Area of Rbm vessels µm ² /mm Rbm*	895 (195- 2159)	882 (0- 2731)	0.8 (NS)	574 (395- 1224)	477 (104- 1902)	0.2 (NS) [¶]
Density of LP vessels No./mm ² †	271 (146)	259 (125)	0.7 (NS)	247 (81)	235 (78)	0.8 (NS) [¶]
% Vascular area in LP µm ² /µm ² x100 [†]	5.8 (2.1)	5.9 (3.2)	0.9 (NS)	5.8 (3.4)	4.8 (1.9)	0.5 (NS) [¶]
No. of vessels in the Rbm stained for VEGF/mm Rbm*	0.6 (0.0- 5.4)	1.6 (0.0- 12.0)	0.9 (NS)	1.0 (0.0- 5.4)	0.0 (0.0- 3.8)	0.8 (NS) [‡]
Area of vessels in the Rbm stained for VEGF µm ² /mm Rbm *	22 (0- 746)	104 (0- 2226)	0.7 (NS)	28 (0- 534)	0 (0-402)	0.8 (NS) [‡]
No. of Rbm vessels stained for TGF-β/mm Rbm*	1.3 (0.0- 8.0)	3.1 (0.0- 13.0)	1.0 (NS)	2.5 (0.7- 4.0)	2.7 (0.0- 8.0)	0.7 (NS) [‡]
Area of Rbm vessels stained for TGF-β µm ² /mm Rbm*	135 (0- 4029)	167 (0- 1519)	0.2 (NS)	255 (42- 311)	181 (0- 1069)	1.0 (NS) [‡]

*Median (range), †Mean (SD), ‡Wilcoxon two-related-samples test, ¶Paired-samples t-test

¹ For within the group differences, NS = not significant

*Number of subjects for FP vs. placebo for Rbm splitting, Rbm vessels and LP vessels measurements: 15 vs. 7, for VEGF stained vessels: 16 vs. 7 and for TGF-β 13 vs. 4

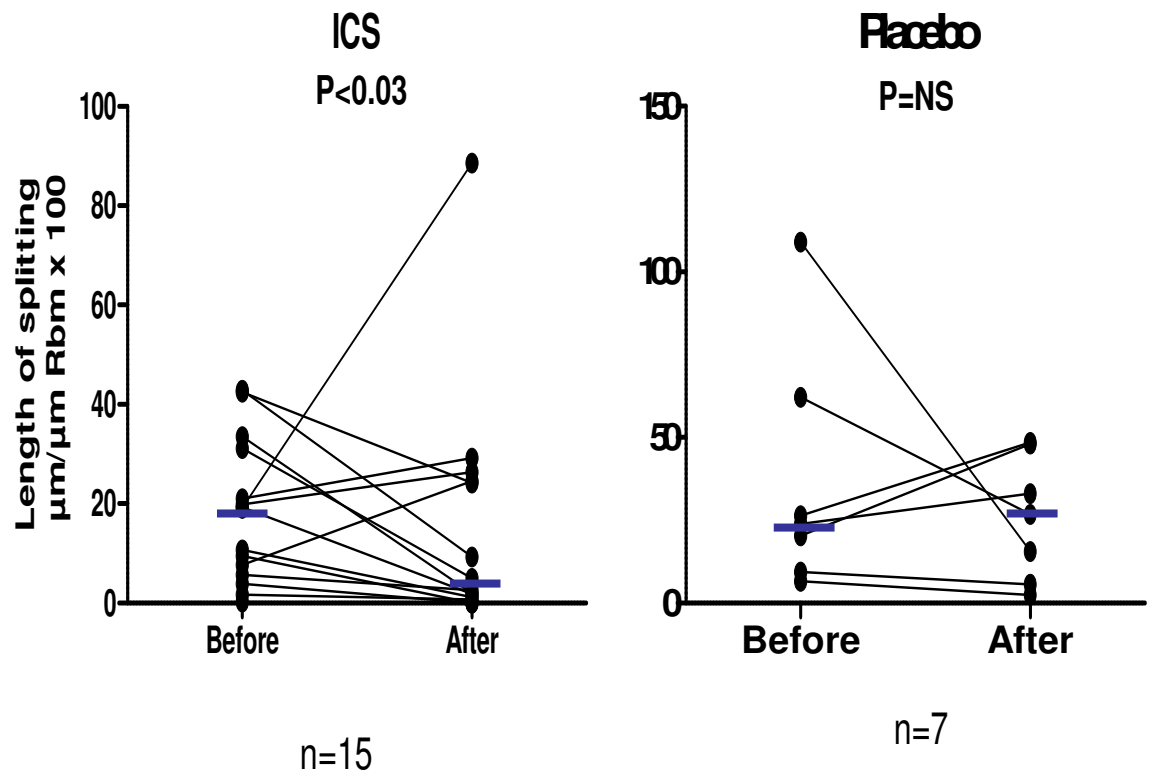


Figure 6-3 . *Rbm* splitting before and after treatment in the two groups. NS= not significant. Bars are medians.

The absolute number or percentage of *vessels stained for VEGF* [median (range) for number of VEGF stained vessels in the Rbm/mm of the Rbm 0.6 (0.0-5.4) before vs. 1.6 (0.0-12.0) after FP, $p = 0.9$ and 1.0 (0.0-5.4) before vs. 0.0 (0.0-3.8) after placebo, $p = 0.8$ and for area of VEGF stained vessels in the Rbm $\mu\text{m}^2/\text{mm}$ Rbm 22 (0-746) before vs. 104 (0-2226) after FP, $p = 0.7$ and 28 (0- 534) before vs. 0 (0-402) after placebo, $p = 0.8$] or *for TGF- β* [median (range) for the number of vessels stained for TGF- β in the Rbm/mm Rbm 1.3 (0.0-8.0) before vs. 3.1 (0.0-13.0) after FP, $p = 1.0$ and 2.5 (0.7-4.0) before vs. 2.7 (0.0-8.0) after placebo, $p = 0.7$; for area of vessels stained for TGF- β in the Rbm $\mu\text{m}^2/\text{mm}$ Rbm 135 (0-4029) before vs. 167 (0-1519) after FP, $p = 0.2$ and 255 (42-311) before vs. 181 (0-1069) after placebo, $p = 1.0$] did not change significantly with either treatment (Table 6-4 and Figure 6-5).

FVC and FEV1 improved in the active treatment group (mean increase 94 ml and 67 ml respectively), but deteriorated in the placebo group (mean decrease -149 ml and -119 ml respectively) (Tables 6-5 and 6-6). The changes in FVC and FEV1 for the FP group compared to the placebo group were significantly different ($p < 0.02$) (Table 6-6). The change in FER (FEV1/FVC %) with treatments was not significant either within or between the two groups [mean (SD) 57 (9) before vs. 57 (10) after FP, $p = 0.6$ and 54 (10) before vs. 53 (9) after placebo, $p = 0.3$; comparison between the two groups for changes in FER $p = 0.4$]. Inspiratory capacity (IC) decreased with FP, but increased with placebo (mean change -93 ml and + 94 respectively). The difference between the two groups was significant ($p < 0.05$). Within-group comparison revealed significant reduction in FVC% predicted with placebo [mean (SD) 116 (15) ml before vs. 111 (17) ml after placebo, $p < 0.05$] but not with FP [mean (SD) 114 (15) ml before vs. 116 (15) after FP, $p = 0.1$]. Within-groups analyses did not show any difference before and after either types of treatment in FEV1, FEV1% predicted, FVC or IC (Table 6-5).

Of those in the FP group, ten of fifteen who had enough tissue for anticollagen IV antibody staining were currently active smokers. Splitting improved significantly with treatment in this group [median (range) 19.6 (1.7-42.8) vs. 3.8 (0-29.2), $p < 0.04$]. There

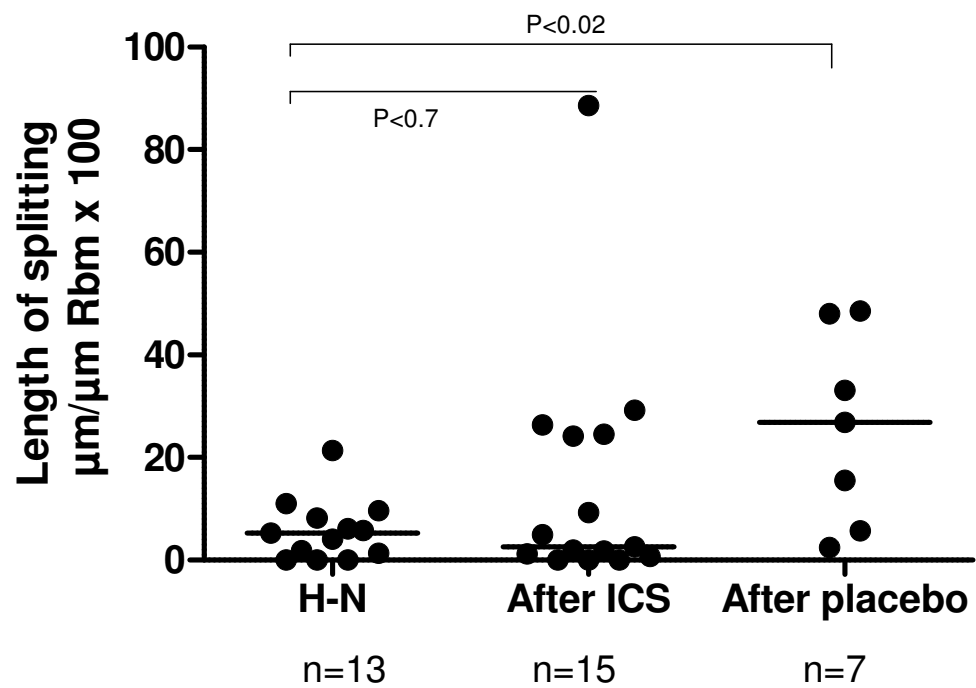


Figure 6-4. Length of splitting after treatment compared to the healthy nonsmoking group (H-N) in the cross-sectional study (Chapter Five). ICS, but not placebo, normalised the length of splitting.

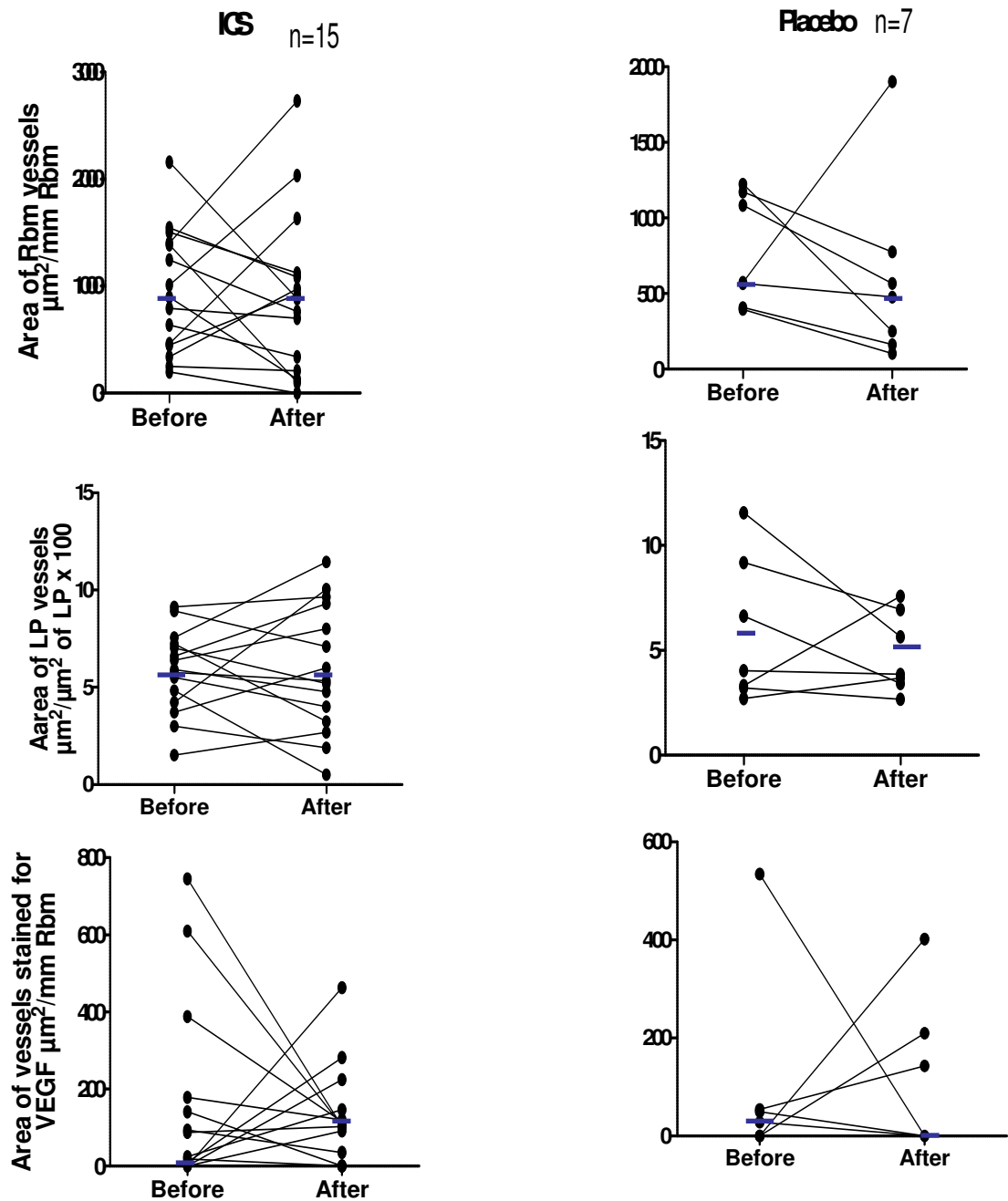


Figure 6-5. Response of vascular remodelling and VEGF to treatment. P not significant for all. Bars indicate median for Rbm vessels and vessels stained for VEGF and indicate mean for LP vessels.

Table 6-5. Within-group changes of lung function measurements with treatments*‡

	Before FP	After FP	P value	Before placebo	After placebo	P value [†]
FEV1, ml	2362 (428)	2429 (430)	0.1	2220 (545)	2101 (578)	0.1
FEV1% predicted	80 (14)	82 (14)	0.06	78 (13)	73 (15)	0.1
FVC, ml	4235 (873)	4329 (897)	0.09	4131 (701)	3983 (739)	0.08
FVC% predicted	114 (15)	116 (15)	0.1	116 (15)	111 (17)	<0.05
IC, ml	3020 (698)	2927 (693)	0.08	2844 (648)	2938 (633)	0.2

*Numbers are mean (SD)

[†]Paired samples t-test ‡Number of subjects for FP =18 and for placebo = 8

Table 6-6. Between-groups comparisons of changes in lung function measurements with treatments*

Groups (numbers)	FP (n=18)	Placebo (n=8)	P value [†]
Δ FEV1, ml	+67 (170)	-119 (175)	<0.02
Δ FEV1% predicted	+3 (6)	-4 (7)	<0.02
Δ FVC, ml	+94 (222)	-149 (202)	<0.02
Δ IC, ml	-93 (215)	+94 (198)	<0.05

*Numbers are as mean (SD)

[†]Independent-samples t-test for between-groups differences

were no changes in the number of Rbm vessels /mm Rbm [median (range) 9.1 (3.1-23.0) before vs. 6.5 (1.2-18.0) after treatment, $p = 0.25$], area of Rbm vessels $\mu\text{m}^2/\text{mm Rbm}$ [median (range) 953 (338-2159) before vs. 903 (100-2731) after treatment, $p = 0.8$], number of vessels in the LP/mm² LP [mean (SD) 234 (141) before vs. 207 (110) after treatment, $p = 0.5$] and area of vessels in the LP ($\mu\text{m}^2/\mu\text{m}^2 \text{ LP}$) x100 [mean (SD) 5.3 (2.0) before vs. 5.3 (3.7) after treatment, $p = 1.0$] with FP in this subgroup analysed independently.

No independent analysis was done for the 5 individuals on FP who were ex-smokers, but empirically their Rbm splitting also normalised; but of course their vessels were not abnormal to start with.

There were no correlations between changes in remodelling parameters and changes in lung function.

Discussion:

This study, as far as my reading suggests, is the first clinical trial examining the effects of ICS on Rbm and vascular remodelling in COPD. I have evaluated the effects of ICS on airway remodelling in mild to moderate COPD and revealed that Rbm fragmentation is responsive to corticosteroid treatment but vessel remodelling and angiogenesis is not.

ICS are part of standard management of a large proportion of COPD patients (P.J Barnes, 1998) (Rodrigo et al., 2009) because of empirical observations in large clinical trials. Reports have shown effectiveness of ICS on airway remodelling in asthma, reducing the thickness of Rbm and vessels in the mucosa in asthma (B. N. Feltis et al., 2007; Olivieri et al., 1997; C. Ward et al., 2002), but this is a very neglected area of investigation in COPD.

Investigations have already reported the effects of ICS on inflammatory changes in COPD (Hattotuwa et al., 2002; D. W. Reid et al., 2008) (Bourbeau et al., 2007). The current study reveals that ICS was effective on reversing Rbm changes but in contrast to

asthma, did not change the number or area of vessels in the airway mucosa. This finding is compatible with the relative resistance to corticosteroids in COPD that is seen in clinical practice. Characteristics of airway inflammation and remodelling are quite different in COPD vs. asthma, and so it seems is the response to ICS. In addition, smoking rather than allergic inflammation is the main aetiology of COPD.

In Chapter Five, I have demonstrated that vessel remodelling in ex-smokers with COPD is not different from nonsmoking normal subjects, suggesting that vessel remodelling is probably responsive to smoking cessation. It is therefore notable that what may be reversible with smoking cessation in COPD is not with ICS therapy.

As discussed in Chapter Two, ICS exert their effects through both genomic and nongenomic mechanisms. It has been suggested that action of ICS on airway remodelling is via genomic mechanisms that need time and involve the regulation of target genes (G. Horvath & Wanner, 2006), but this needs specific investigation in COPD airways.

It has been suggested that COPD is “resistant” to ICS (P. J. Barnes & Adcock, 2009; To et al., 2010). There are some reports that did not find any effects with ICS on clinical manifestations and airway inflammatory changes in COPD (Culpitt et al., 1999; Keatings et al., 1997; Rodrigo et al., 2009; J. Vestbo et al., 1999). However, most previous studies support at least limited efficacy of ICS in patients with COPD. Improvement in symptoms and in quality of life, reduction of the rate of acute exacerbations and improvement or stabilisation of lung function parameters have been reported in many studies (Aaron et al., 2007; Burge et al., 2000; P. Calverley et al., 2003; P. M. Calverley et al., 2003; Peter M. A. Calverley et al., 2007; Choudhury et al., 2007; Hanania et al., 2003; Mahler et al., 2002; Paggiaro et al., 1998; Pauwels et al., 1999; D. W. Reid et al., 2008; Singh et al., 2008; Szafranski et al., 2003; TLHSRG, 2000; Verhoeven et al., 2002; Wedzicha et al., 2008; E. F. M. Wouters et al., 2005). Improvement in inflammatory markers in the airways of COPD subjects has also been

reported (N. C. Barnes et al., 2006; Geczy et al., 2002; Hattotuwa et al., 2002; D. W. Reid et al., 2008) (for more details see Chapter Two, Section 13.c use of ICS in COPD).

Pulmonary function testing showed improvement in FEV₁, FVC and IC with FP and deterioration with Placebo. Changes of lung function before and after treatment were significantly different between the two groups. These results were previously published by Reid et al. (D. W. Reid et al., 2008). Reid et al. also reported that ICS reduced neutrophils in BAL and macrophages, CD8⁺ and mast cells in BB.

The failure of ICS in my study to reverse the vascular components of remodelling could perhaps be regarded as relative steroid resistance. But this is unlikely to be due to poor delivery of the drug to the airway. The fact that in stable COPD systemic steroids were not more effective than ICS supports this (J. Vestbo et al., 1999). We used dry powder FP in our study that has been shown to be reliable to adequately deliver drugs to the airways (Morice, Peterson, Beckman, & Osmanliev, 2007) (Schulte et al., 2008) (D. S. Wilson, Gillion, & Rees, 2007).

Where it occurs, relative resistance to corticosteroids may be related to the reduced expression of histone deacetylase-2 (HDAC-2) in COPD (see Chapter Two for details). HDAC is a repressor of production of proinflammatory cytokines. Interestingly this enzyme is adequately present in asthma. Corticosteroids need this enzyme to inactivate inflammatory genes (P. J. Barnes, 2009; Ito et al., 2005). Barnes et al. (P. J. Barnes & Adcock, 2009) have suggested from molecular studies mainly (Ito et al., 2005; Ito et al., 2001), that HDAC activity is decreased in COPD airways, but this has not taken into account the major alterations in airway cell populations present. Further work on this is required.

There were no significant correlations between changes in airway remodelling and changes in lung function parameters. COPD is a complex syndrome made up of a combination of alveolar septal destruction, small airway and large airway disease (Hogg & Pierce, 2008) (Hogg et al., 2004) (A. Nagai, West, & Thurlbeck, 1985) (Suisse &

Barnes, 2009). In this study I have only examined the effects of ICS on remodelling in the large airways. In the airway component most of the physiological defect is said to be focused in the small airways (Hogg et al., 1968) (Hogg et al., 1994; Snider, 1985), and we did not have immunopathological data on this. A placebo-controlled trial compared the effects of ICS (budesonide) with placebo in the progression of emphysema in current smokers with COPD for 2-4 years and did not find significant protection by active treatment. The investigators used annual CT-scans to detect the progression of emphysema (Shaker et al., 2009).

As the effects of smoking on alveolar septae look irreversible at this stage, we probably need to examine small airways to find the mechanisms of improvements in lung function in our COPD subjects. This is not easy to do in patients with COPD using bronchoscopic methods. Even so, changes in inspiratory capacity (IC) support the idea that change did occur in that compartment. Thus IC reduced with FP but increased with placebo, there was a significant difference between the two methods of treatment (Table 6-6). This probably means that FP reduced hyperinflation as an index of small airway disease. Since COPD is most probably a pan-airway disease with common pathological changes throughout the airways (large and small), as it is in post-transplant bronchiolitis obliterans syndrome (BOS) where the physiological impact is also in small airways (Snell et al., 1997; C. Ward et al., 1997; Chris Ward et al., 1998; Ling Zheng et al., 2005) this improvement in IC is very likely to mean that ICS had positive effects there. Whether this change was on inflammation alone, or also on remodelling in that site, needs further studies to elucidate.

In *summary* this study has for the first time shown that ICS improved Rbm splitting and fragmentation but did not change vessel remodelling in the mucosa of large airways in COPD, nor it did change vessel-associated VEGF and TGF- β activities in the Rbm. There were no correlations between lung function and pathological changes. It is likely that ICS in our study had positive effects on both large and small airways in COPD patients, but further studies specifically sampling the latter compartment are needed to clarify this.

Chapter Seven

Summary and General Discussion

1. Chapter One-Preliminary study:

Remodelling in inflammatory diseases of the airways has been under vigorous investigation during the last two decades. The characteristics of remodelling, particularly vascular remodelling have been addressed in quite a few of published reports. However, the gap between the research effort on airway remodelling in asthma and COPD is obvious, COPD being well behind (E. Haydn Walters et al., 2010). The Respiratory Research Group in the Menzies Research Institute, University of Tasmania decided to examine the bronchial biopsies (BB) available from tissue bank of COPD subjects for remodelling changes.

While doing a preliminary observational study of slides from tissue samples taken by bronchoscope from COPD subjects Mr. Sukhwinder Singh Sohal, a fellow PhD student and I noticed interesting changes in the Rbm that to our knowledge have never been reported before. The Rbm was fragmented with clefts within it. The first question at that time was whether these changes were artifact or not. A quick look at available tissues from our group's previous studies on asthma and normal controls revealed that they most probably were not artifact of tissue processing, which is standard. Later, the results of my preliminary, pilot study confirmed that these changes are either related to COPD or to smoking, or both.

The second question was how to measure and report fragmentation of the Rbm. Later in our cross-sectional studies both Mr. Sohal and I measured Rbm fragmentation independently for our purposes using different staining and quantitation methods. Independently, both of us found significantly greater fragmentation in COPD compared to a normal control group. Current smokers with physiologically normal lungs also had increased fragmentation of the Rbm. I also noted changes in the distribution of vessels in the airway mucosa, initially focusing on vessels that were in contact with and penetrating within the Rbm.

To start, I compared BB from current smoking COPD subjects with healthy nonsmoking volunteers. I measured the length of clefts or splits (please see Chapter One) as a surrogate for fragmentation of the Rbm and I used specimens stained with Collagen IV antibody to study vascular remodelling.

To investigate the difference in tissue distribution of vessels I divided the LP into three discrete compartments. First Rbm, then the LP to the depth of 20 μm from the Rbm and third the rest of the LP to a depth of 150 μm from the Rbm and compared the number and area of vessels within these three compartments between the study groups. For the Rbm, I measured and analysed separately three types of vessels that I was able to discriminate during my observations of the tissue from COPD subjects: those vessels that contacted the anti-luminal border of the Rbm, those that apparently were penetrating the Rbm and those that were completely embedded within the Rbm. The 20 μm measurement was non-revealing but the analyses of Rbm vessels and for the whole LP up to 150 μm , revealed new aspects of vascular remodelling in COPD that to my knowledge have never been reported before in smokers or in COPD. For the rest of my work, I found the detailed anatomical classification of Rbm vessels too complex, and decided to simplify the analyses as Rbm-associated vessels against LP vessels. These vascular changes led to investigation of the potential role of angiogenic factors in the airways of the same subjects.

2. Chapter Two- Literature review-Themes:

Most of the available literature addressing airway remodelling is about asthma, although there were reports about structural changes in the airways in smokers decades ago. Recognising early pathogenetic mechanisms and susceptibility factors are of vital importance in preventing disease evolution or progression.

Most studies on COPD have used autopsy specimens or lung resections from subjects undergoing surgery for lung cancer. Emphysema and small airway pathology were the focus of these studies. Even the “normal” control volunteers had been operated for the same reason. In contrast to asthma, not many studies in COPD/smoking have used BB to examine the characteristics of airway remodelling in large airways in COPD. These few studies (Calabrese et al., 2006) (Zanini et al., 2009) have recruited a subgroup of COPD patients that cannot be considered as typically representing the COPD population seen in daily practice.

The aetiological association between remodelling and inflammation is another subject that needs research. Their cause and effect relationship is not clear yet. Oxidative stress, inflammation, epithelial-mesenchymal transition (EMT) and remodelling may be inter-related, as they are driven by common intracellular mechanisms and can accentuate each other.

Vessels presumably play a key role in airway inflammation and remodelling. They are the port of entry for cells and cytokines and also deliver growth factors and nutrients to tissue. Regulatory mechanisms of vessel formation are very complex and involve many mediators. In general, investigations on this subject are very active, but in COPD itself they are still very limited.

Corticosteroids are frequently used in the management of COPD. There are many reports on their effect on clinical manifestations and also on airway inflammation in COPD subjects. To our knowledge, there is no published clinical trial yet about the effects of ICS on airway remodelling in COPD.

3. Chapter Three (General Methods)- Methods, subjects and tissue:

BB had been taken by the clinical team I was working with from study subjects before I started my PhD. Of specimens taken from 65 subjects that I had available, only 59 and 63 specimens, respectively, had enough remaining tissue for Collagen IV and VEGF cross-sectional studies. The problem with TGF- β was broader; many samples were so densely stained that no measurement was possible, even diluting the antibody to 1:16000 could not fix the problem, so only 45 biopsy specimens could be examined for TGF- β . However, power calculations and sample size estimation revealed that we had enough subjects for an adequate cross-sectional study.

I encountered the same issues with the longitudinal ICS-intervention study, which had been completed clinically before I started my PhD. From 34 subjects who volunteered for the study, I found that there were 26 potentially usable pairs of tissues; but for various reasons (see Chapter Six) only 22, 23 and 17 pairs of biopsies had adequate tissue to be included in the studies using Collagen IV, VEGF and TGF- β staining, respectively. The study was designed to randomly allocate study subjects 2:1 into respectively active and placebo treatments. This design was to keep enough subjects in our active arm for acceptable statistical power, emphasising the importance of within-group analysis. My retrospective sample size calculation revealed that there were enough samples for all the before and after comparisons, except for the LP vessels. Even so, there was no suggestion in the data that we had a Type-2 statistical error in this latter analysis.

One potential ethical issue that may be raised about our longitudinal study was that the subjects who were smokers needed to continue smoking throughout the study to stay in

the study. During recruitment all smoking volunteers were offered smoking cessation therapy. Only those who did not want to stop smoking were recruited into the study. A potential technical problem was the antibody we used to stain vessels, that is anti-Collagen IV antibody. This antibody has been used to delineate vessels in the airway mucosa in many studies, including studies published by our group (Barsky et al., 1983; B. N. Feltis et al., 2007; Bryce N. Feltis et al., 2006; Li & Wilson, 1997; Orsida et al., 1999; Orsida et al., 2001). However, it is always possible that such immunostaining is not sensitive enough or is not specific for vessel structures. I did a tissue-matched study to compare staining of Collagen IV with staining for Factor VIII, to ensure that the structures that we have been measuring are really vessels. Comparing slides from the same subjects confirmed that the structures were indeed vessels.

Measurement of vessels in the Rbm was another technically challenging subject for me. Obviously, all the measurements needed to be normalised. For splitting it was straightforward and I simply divided the length of splits by the length of Rbm that was examined. The question was what the best method would be to normalise the number and area of Rbm vessels. I chose to normalise these measurements by dividing them by the length of the respective Rbm. I did not choose the surface area of the Rbm for this purpose because of the following reasons: First, the Rbm was a linear structure in some samples and therefore did not have any measurable surface area. Second, the anti-luminal border of the Rbm was not well defined in some samples and therefore, I could not discriminate it from the surrounding soft tissue, which would make the calculation of Rbm surface area imprecise and only at best an estimate. Third, in the samples from smokers and COPD subjects there was Rbm fragmentations that in some samples made large holes within the substance of the Rbm. For all these reasons I decided to divide the number and area of vessels by the length of the Rbm to correct my measurements and make them comparable between subjects. I believe that Rbm thickness is probably abnormally variable in COPD, but on formal testing of my results repeatability and reproducibility were acceptable (see Chapter Three).

I chose to measure LP vessels to the depth of 150 μm into the LP from the Rbm. This proved to be acceptable, although the thickness of the LP was variable in the biopsies, and dividing the measurements by the area of the LP available and examined corrected for this potential problem.

One of the problems with studies that use human sample is relative shortage of volunteers. This is especially true for the studies that need tissue samples obtained by invasive procedures like bronchoscopy. Therefore, frequently it is hard and very time-consuming for investigators to recruit enough subjects. That is why most studies that examine BB do have small sample size and are prone to false negative results. My retrospective sample size calculations indicated that there were enough subjects in each one of our study groups in my cross-sectional study to avoid Type-2 statistical errors. Compared to other studies that have used BB (e.g. Zanini et al. and Calabrese et al.) (Zanini et al., 2009) (Calabrese et al., 2006), the size of our sample was robust. For my longitudinal study, all parameters, except for vessels in the LP, had sufficient sample sizes for detecting important treatment effects.

4. Chapter Four (Methods Part 2)-Histochemical staining of vessels:

This study was designed to compare anti-Collagen IV with anti-Factor VIII antibodies for immunostaining of vessels. The results not only showed quite interesting differences between the two methods of vessel marking, but also supported our previous findings in the cross-sectional study: Factor VIII antibody staining of the vessels in the LP confirmed that there was reduced vascularity in smokers with COPD compared to control. In this study I assessed the agreement between the two methods of vessel staining by correlation graphs and then by the method of Bland and Altman. Anti-Factor VIII antibody was revealed to be more sensitive than Collagen IV antibody in detecting smaller vessels, but Collagen IV antibody marked vessels with larger cross-sectional area more efficiently.

5. Chapter Five-The cross-sectional study:

This large piece of work rejected my hypothesis that airway remodelling findings from the preliminary studies were specific for COPD. However, some remodelling changes were exaggerated in current smoking COPD suggesting an interaction. The cross-sectional study showed that vessel changes started in smokers before deterioration of lung function; this is compatible with previous reports finding inflammatory changes in the airways of smokers with normal lung function (Hogg et al., 1994) (Hogg & Timens, 2009) (Fletcher & Peto, 1977). Moreover, none of the vessel changes I found in the current-smoking groups compared to normal nonsmoking subjects existed in the ES-COPD group. This is in contrast to the findings for Rbm fragmentation. Absence of difference between ES-COPD and H-N may suggest that smoking cessation may reverse remodelling changes; but, as this study was not a longitudinal smoking-quitting one, I could not make this conclusion definitively. We now do need such a longitudinal smoking-cessation study.

The finding of Rbm fragmentation raised the possibility that I was seeing the effects of proteolytic enzyme cleavage on the Rbm. Our group hypothesised that smoking may activate epithelial-mesenchymal transition (EMT). My fellow PhD colleague, Mr. Sohal, has tested this hypothesis and his findings so far strongly support the idea that EMT is active in smokers and especially in COPD subjects.

Compared to recently published studies (Zanini et al., 2009) (Calabrese et al., 2006), my observations on vascular remodelling in smokers are different. I found hypovascularity of the LP in current smokers with or without COPD compared to normal controls. There was no evidence for increased angiogenic activity in the LP of the current smoker groups. In contrast, my study showed hypervascularity of the Rbm in current smokers and increased Rbm-associated vessels stained for VEGF and TGF- β in both current smoker groups and also the ex-smoker COPD group. Although I separated the mucosal vessels into two compartments and measured them separately, this does not explain completely the differences between my data and previous reports. As previously

explained in the Discussion in Chapter Five, differences in methodology and subject selection may explain some of this apparently contrast.

The finding of hypovascularity in the LP of current smokers, which I believe is real, is especially interesting and is supported by older investigations I have found which used injected dye to study bronchial arteries in the airways in COPD subjects (Cudkowicz & Armstrong, 1953; Paredi & Barnes, 2009). Cudkowicz and Armstrong were interested in the bronchial arteries in post-mortem investigation of patients with emphysema. They included 18 cases who had died of COPD. A radio-opaque medium containing bismuth was injected into the aorta to visualise the bronchial arteries radiographically, and with the presence of bismuth it was possible to examine bronchial artery branches histologically in the airways. Their study found narrowing and obliteration of bronchial artery branches to be widespread in both radiological and pathological assessments (Cudkowicz & Armstrong, 1953).

In contrast to Calabrese et al., I found some correlations between vascular remodelling and lung function, indicating a potential physiological significance for my findings. The current smoker COPD group in my study had positive correlations between FEV1% predicted and Rbm vessels stained for VEGF and also between LP vessel number and FVC% predicted. It is also notable that there may be some reversibility in this vascular manifestation of COPD with quitting cigarettes. It would be interesting in a planned prospective smoking-cessation study to relate vascular changes over time with any improvement in lung function.

An independent measurement of vessels with Factor VIII staining confirmed hypovascularity of the LP in COPD subjects (see Chapter Four). As explained in Chapter Five and Chapter Four, I have presumed that our finding of fewer vessels in the LP of COPD subjects indicates reduced angiogenic activity. However, my study was not able to show reduced activity of VEGF in smokers, but further studies on angiogenic/antiangiogenic factors or markers of endothelial proliferation such as integrin $\alpha v \beta 3$ (Calabrese et al., 2006) are now indicated.

6. Chapter Six-The longitudinal study:

This showed that Rbm fragmentation was responsive to ICS therapy but not placebo. However, vascular remodelling did not change with treatment, neither did vessel-associated VEGF or TGF- β expression in the Rbm. To my knowledge, this is the first longitudinal study that has evaluated the effects of ICS on vascular and Rbm remodelling in the airways of COPD subjects. This study, like studies that have assessed the effects of ICS on clinical manifestations and inflammatory markers (N. C. Barnes et al., 2006; Burge et al., 2000; Peter M. A. Calverley et al., 2007; Paggiaro et al., 1998; Pauwels et al., 1999; D. W. Reid et al., 2008), indicates that there are steroid-responsive components in COPD, although clinical effects are not as dramatic as in asthma. This is not surprising because these two diseases are quite different in many aspects including the details of airway remodelling. On the other hand, this study alongside many other clinical trials supports the modification of the traditional definition of COPD emphasising its potential treatability. Indeed, GOLD did recently add the word “treatable” to the definition of COPD (GOLD, 2009). My studies added strength to this decision, and this insight might change the nihilistic attitude of many clinicians towards this disease.

Current guidelines by international organisations like GOLD state that severe COPD patients with frequent exacerbations benefit from ICS (GOLD, 2009). However, our subjects suffered from mild to moderate COPD and therefore, according to such guidelines may not have an acceptable risk-benefit ratio for long-term treatment, especially with high dose of FP. COPD patients are usually old and high dose ICS is not without systemic side effects in these patients. Nevertheless, we know that many COPD subjects with milder forms of COPD use these medications in clinical practice (Choudhury et al., 2007; Rodrigo et al., 2009). We probably now need a study to see if we can obtain the same structural improvement with lower dose ICS, and whether long-term advantage ensues as a direct consequence.

One may question the value of examining large airways, where the major compartment causing physiological obstruction in COPD is in the small airways. Our findings which did not show any correlations between the changes in remodelling and lung function parameters with treatment may strengthen this critical view. However, for the reasons below our Research Group chose to use large airway biopsies for the longitudinal study:

- As said before, COPD is a pan-airway disease (Cosio Piqueras & Cosio, 2001; Hattotuwa et al., 2002; Hogg, 2004; Hogg, 2008) (Steven D. Shapiro & Ingenito, 2005). The similarity of pathological changes in airways of different sizes in diseases with widespread airway involvement, such as post lung transplant bronchiolitis obliterans syndrome (BOS) has been demonstrated (Snell et al., 1997; C. Ward et al., 1997; Chris Ward et al., 1998; Ling Zheng et al., 2005).
- Sampling of large airways is easier and safer than for small airways. For the latter, you usually need to look for subjects with COPD who are undergoing thoracotomy and surgery for some reasons, but this invariably means that they have another disease (usually cancer) in close proximity which may be confounding. Similarly, this also means that you cannot have a true normal control group. In many studies that have used resected lung tissue, the individuals in control group, even if they had lung function within normal limits, also had localised lung disease that was serious enough to make them appropriate candidates for surgery. Again, this could lead to some element of confounding. And of course, it is hardly possible to repeat sampling before and after treatment to compare the results of your research intervention. On the other hand, doing any type of invasive procedure for COPD subjects who suffer from something more than mild to moderate disease means taking risks with their safety. This is particularly true for procedures such as thoracic surgery, but also for bronchoscopic trans-bronchial biopsies. With the latter procedure, the

amount of small airway tissue obtained is usually fragmentary at best, and rarely suitable for quantitative analysis.

7. Final Summary and Conclusions:

My studies have shown novel Rbm and vascular changes in BB from smokers with or without COPD. Compared to previous reports in asthma, these changes are quite different. Asthma is characterised by Rbm thickening and hypervascularity of the LP, the latter is related to the increased activity of the angiogenic factor VEGF. In contrast, the Rbm is not uniformly thicker than normal in COPD, but it is nonhomogenous and fragmented. These changes are smoking-related but are evident in COPD even after quitting. The Rbm is hypervascular in BB from smokers with COPD but the LP is hypovascular. These changes are also smoking-related and probably responsive to smoking cessation, as they were not present in my ex-smoker COPD group. However, a longitudinal smoking cessation study is now needed to test this hypothesis. The cross-sectional study did not find any meaningful relationship between VEGF positive or TGF- β positive vessels and the number or area of vessels in the Rbm or LP in our COPD subjects. In contrast to asthma, there was no change in vascular remodelling nor angiogenic factors with ICS treatment. However, there is a common feature between asthma and COPD; in both Rbm changes are responsive to ICS. It is interesting that this happens despite continuing smoking in COPD subjects. Therefore, airway remodelling in COPD, as for airway inflammation and clinical manifestations, is to some extent responsive to ICS, but quantitatively and qualitatively different from the steroid responsiveness of asthma.

My studies might have answered some questions, but have raised even more! For example, what is the reason for Rbm fragmentation? Why is it responsive to ICS therapy despite being smoking-related? Why is the Rbm hypervascular and the LP hypovascular in smokers with normal lung function and COPD? Why is vascular remodelling resistant to ICS? A longitudinal study will reveal which parts of airway remodelling are

responsive to smoking cessation. Other angiogenic factors and markers of angiogenesis need to be studied to find the underlying mechanism of the vascular changes in smokers. Further investigations on epithelial-mesenchymal transition (EMT) and evaluation of its response to ICS may clarify some questions about Rbm fragmentation. And finally, a more thorough investigation on the complexity of inflammation in the airways of smokers and COPD subjects and the relationships between these and remodelling changes is highly desirable.

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Appendix: Examiners' Comments

Examiner 1

Major concerns:

Comment 1.

Chapter 6 seems almost completely out of place. It is basically a comparison/validation of methods rather than a results chapter and belongs within an extended Chapter 3. Indeed on p 267 the candidate refers to a study comparing immunostaining for collagen IV and Factor VIII as part of Chapter 3, suggesting that this is where the material in Chapter 6 was intended to be located, or had been located in an earlier version.

Answer:

I am very happy to make this chapter more a part of Methods; I placed it where it is because it turned out to be very interesting data which related to the likely 'maturity' of vessels in the subepithelial lamina propria in COPD which is consistent with the fewer but larger vessels seen there, i.e. relative decrease in angiogenesis, which is quite a novel and potentially important finding. But I agree that essentially the chapter is about a comparison of Methods. To fully amalgamate with the current Methods Chapter (Three) would make this a very long Chapter Three, so I have renumbered the chapter to make it Chapter Four, but emphasizing that it is a second but more specific and applied Methods section. I have added a new introduction to this new chapter (page 173).

Comment 2.

The strong background staining for TGF- β , even at very high dilutions, suggests that much of the apparent immunoreactivity that was quantified might

have been non-specific staining, which raises significant concerns about the interpretation of these data. The problem is not explicitly acknowledged or discussed.

Answer:

We feel that it is more likely that there is just a lot of TGF- β in many sections. But this was variable and some sections stained much less, suggesting specificity at the preparation were all the same. Further, my counts were specifically directed only at vessels in which endothelial cells were seemed stained with TGF- β , albeit again variably. I do not feel that any further comment within the chapter is needed.

Comment 3.

There is potentially a serious error with respect to statistical methodology for comparison of more than 2 groups. On p 164 the candidate states that “To test the difference between 2 groups, Student’s t-test was usedWhen more than 2 groups were compared, first ANOVA was used to test if there was any difference among the groups. If a significant difference was found, post-hoc use of t-test was used (sic) to compare the likely groups for difference.” If this is actually what was done, i.e. use of Student’s t-test following ANOVA, it was inappropriate. In a multiple comparison analysis, selected groups can be compared using a Bonferroni’s corrected t-test, but the text does not indicate that a corrected t-test was performed. If not, the data need to be re-analysed.

Answer:

I do not believe that there has been an error in the statistical analysis. I took statistical advice before doing this analysis and also again now since receiving the examiners’ comments, and I am fairly sure of my ground in this. However, my description of the method of analysis was somewhat at fault and may have misled the examiner. Specifically, I referred to the post-ANOVA tests of differences between pairs of means as being “post-hoc”, when in fact these comparisons were determined a priori in the design of the experiments reported in Chapter Five. To be clear about this, the experiments were designed to be a comparison of COPD groups

with the non-smoking control group. A smoker control group was added at the design stage to test whether the differences observed could be attributed to smoking. Thus, the comparison of the COPD groups with the non-smoking controls, and of the smoker controls with the non-smoking controls, were specified as a hypothesis for the studies reported in these thesis (please see Chapter One, Introduction, Preliminary Data and Resulting Aims and Hypotheses, page 4 and 31). In these circumstances, the recommended statistical approach is to perform the contrasts without Bonferroni's adjustment or any other multiple comparison procedure. For example, McPherson (McPherson G. Applying and Interpreting Statistics: a Comprehensive guide. 2nd Edition, New York: Springer-Verlag, 2001) recommends that “.....These comparisons should be made independently of a general test of the hypothesis that all treatment effects are equal” i.e. the ANOVA (p 414). Indeed my decision to perform an overall test of difference among the groups prior to testing contrasts between groups that were specified *a priori* is a stronger approach than that recommended by McPherson, and should help to reduce the probability of type-I error.

To rectify the error of terminology that was made, reference to post-hoc comparisons has been removed. This was true temporally, but not in design. In particular the sentence on page 164 of Chapter Three, Material and Methods “When *more than 2 groups* were compared, first ANOVA was used to test if there was any difference amongst the groups. If a significant difference was found, post-hoc use of t-test was used to compare the likely groups for difference” has been replaced with “When *more than 2 groups* were compared, first ANOVA was used to test if there was any difference amongst the groups. If a significant difference was found, a t-test was used to compare the likely groups for difference as defined *a priori* in the study hypothesis.”

Comment 4.

Chapters 4 and 5 are presented as if they were independent papers, with redundant Abstracts and a Methods section that says nothing useful in Ch 4,

other that to refer the reader back to earlier chapters. These two chapters should be edited to become part of the continuum of the text of the thesis.

Answer:

This refers merely to just one line in Chapter Five (previous Chapter Four) and a short paragraph in Chapter Six (previous Chapter Five), which are meant to refer the reader back to the main Methods section where the details of the study design are given. This was done like this so that anyone subsequently reading the thesis, especially if reading just each one of these chapters would have helpful directions to the detail of the experimental work. On re-reading the sections I do not agree that there is appreciable duplication and feel that the purpose of the brief entries is valid. Although I do not believe that this is a major or even significant issue, I would prefer to leave this text intact.

Other concerns:

Comment 1.

On p 17 the candidate refers to the surface area of vessels. This surely refers to the cross-sectional area.

Answer:

I agree that this would be better phraseology, and “Surface area” on page 17 of Chapter One is changed to “cross-sectional area”.

Comment 2.

Neither on p 20 nor at any later point in the thesis is an explanation offered for the choice of VEGF and TGF- β for immunostaining, while excluding other possible angiogenic factors e.g. FGFs.

Answer:

VEGF and TGF- β are the best known and most studied mediators of angiogenesis, and most likely to be implicated in a chronic inflammatory condition such as the airway component of COPD. We could not study all mediators (there are at least 12 mediators with angiogenic activity described in the literature) and had to prioritise. I have now made this point as suggested in the text of the Discussion on page 30 of Chapter One.

Comment 3.

On p 26 there is no discussion of the effect of manual setting of the software threshold (“The tissue stained with VEGF antibody was defined by me.”) on the data collected, or of measures taken to minimize operator effects.

Answer:

To minimize the operator effects I used these strategies:

- I was tutored by MRI Hon fellow Dr. Chris Ward, our chief laboratory technician (Mr. Steve Weston) and the representative from Media Cybernetics Inc. (Mr. Con Saponis) to use the software correctly before starting examination of slides. All three are experienced in using this software and Dr. Chris Ward has published several papers using this software.
- I defined the colour of interest using Eyedropper Tab in the Segmentation dialog box of the Count/Size application of the software. The colour of interest was determined for each end-point stain in consultation with Mr. Steve Weston who supervised all steps of processing and antibody staining of tissue. I selected the areas that were definitely and purely stained with the antibody of interest. The sensitivity setting was 4 (out of a scale of 1-5 where 1 is the least sensitive and 5 is the most sensitive) and the Drop colours text box was 1 pixel (which means that every colour cube element containing the selected colour was counted; in other words, no colour cube element containing the colour of interest was considered as noise) and the Expand Selection text box was 1 colour index (out of scale of 0-10, which determines

the broadness of the area selected by the eyedropper; 1 colour index means that only the colour under the eyedropper is selected as the colour of interest; in other words, the area was selected very specifically).

- To ensure methodological consistency and once optimised for our samples, I did not vary the settings of the software.
- I defined the “area of interest” (e.g. the lamina propria) by physically selecting it using the software selection tools available, thereby, avoiding nonspecific counting of other areas.

The sentence on page 26 of Chapter One is changed from “The tissue stained with VEGF antibody was defined by me” to “The tissue stained with VEGF antibody was defined to minimize nonspecific background staining. In summary, the software was set as sensitivity of 4, Drop colours of 1 pixel and Expand Selection of 1 colour index. These settings remained consistent throughout analysis of all slides, and seemed empirically to be good robust solution.”

Comment 4.

On p 37, surely more recent ABS figures than 1998 are readily available?

Answer:

That was the most recent I have been able to find. It is certainly unlikely that the basic ‘message’ at least has changed since 1998

(please see Australian Institute of Health and Welfare, AIHW, available at

http://aihw.gov.au/cdarf/data_pages/mortality/index.cfmhttp://aihw.gov.au/cdarf/data_pages/mortality/index.cfm).

Comment 5.

Similarly on p 66, the 1999 references for the use of anti-VEGF are obsolete.

Answer:

I meant to imply that anti-VEGF therapy was introduced in 1999, but has now indeed become established as a treatment for cancers; I have added a recent citation

to emphasise this (Tol J, Clinical Therapeutics, 2010, 32:437-53) (page 66, Chapter Two).

Comment 6.

The literature review sometimes becomes very tedious as individual papers are reviewed in turn (e.g. pp 105-129) when a summary would suffice for the purpose of the research being performed.

Answer:

There are summary tables on pages 107-112 and 123-125 as a quick reference to this complex area. I thought it is useful to make specific comments about how these studies differ from my work in the thesis. I still think that this is quite useful, and for the fastidious future reader I would prefer to leave them there: it was a lot of work, which I edited a number of times to try and make readable .

Comment 7.

The exhaustive detail for the immunostaining procedures (pp 146-151) is unnecessary.

Answer:

I think it is quite important that future readers know specifically what I did, especially if they are relatively inexperienced in laboratory techniques, or if these methods change substantially over next few years.

Comment 8.

On p 203 it might have been worth considering whether Rbm spitting was induced by the bronchial biopsy procedure, which is known to cause many histological artefacts-this would not affect the validity of the observations but would minimize the implication that such splits exist in vivo.

Answer:

I think that splitting is very likely to be present in vivo because we found vessels and many cells in the clefts produced by this splitting. Further, our group has undertaken many biopsy studies in different disease groups, always with similar techniques and has never encountered these changes before. We believe that they are real biological changes, and not an artefact.

Comment 9.

On page 242 a paragraph is repeated.

Answer:

Sorry, an oversight in editing. The duplicate paragraph on page 242 is deleted.

Comment 10.

It is disappointing that there is no real discussion of the possibility that reduced numbers of inflammatory cells, which are potentially a major source of extracellular proteolytic enzymes, might account for the reduction in Rbm splitting in patients who had been treated with inhaled glucocorticoids. Only in the very last sentence on p 274 does this seem to be acknowledged.

Answer:

In a previous publication we showed that there is not a great change in inflammatory cells in the airways in COPD with inhaled corticosteroids (Reid D, et al. 2008, *Respirology*, 13: 799-809). Further, we have now determined that the cells in the fragmented Rbm are not inflammatory species, but are epithelial cells in transition to mesenchymal cells as part of the process known as EMT. I did not have these data available to comment on when the thesis was written.

Examiner 2

Comment

The background chapter is a good summary of the literature but is far too long. 25% of it could easily be removed.

Answer:

I thank the examiner for the positive opening comment on the literature review. I agree that it is rather long, but I wanted it to be comprehensive and the area I am dealing with is very large and quite complex, clinically, physiologically and immunologically. It was a lot of work getting to grips with all this material and I would prefer not to have to cut it down at this stage. It will be useful background reading for other researchers over the next few years at least, until the field changes too much.

Comment

It would be better if the pilot data and aims followed it rather than vice versa.

Answer:

I do not agree with this comment, and my supervisor and I spent quite a lot of time coming to the design of how to present the flow of work rationally and readably. The pilot study very much directed what went after, including the balance of the literature review.

Comment

The methods chapter contains too much unnecessary detail on pages 140-150. All of the diagrams in this section could be dropped.

Answer:

This comment has already been dealt in the answers to the first examiner.

Comment

This thesis contains three experimental chapters the first two (chapters 4, 5) of which provide interesting information on the airway remodelling changes which occur in asthma and COPD. These 2 studies run together in a logical manner and are complementary.

I contrast the 3rd experimental chapter (chapter 6) does not really add much.

Answer:

I thank the examiner for these positive comments on the structure of the data chapters. Examiner 1 also commented on the positioning of Chapter Six, and as discussed above, this has been incorporated into a second evaluative Methods section. However, I think that the information provided in this chapter is very important and very much relevant to vascular remodelling; I am currently writing the data up as a separate paper.

Comment

The studies appear to have been thoroughly performed and the findings seem robust and stand up to scrutiny individually.

Answer:

I thank the examiner for these very positive comments.

Comment

The results are analysed appropriately.

Answer:

Thank you; I agree, and as stated above, I have re-checked this with a professional statistician after the specific comment of Examiner 1.